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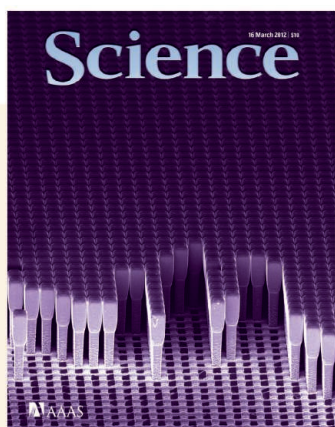
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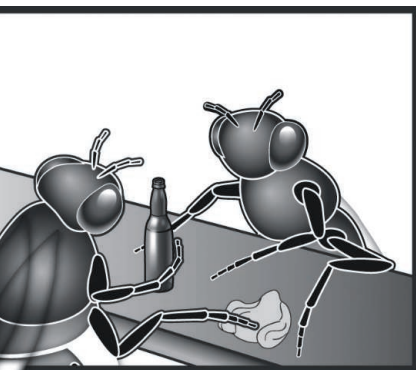
COVER

False-colored scanning electron micrograph of ~8-micrometer-tall germanium crystals, separated by finite gaps, grown onto silicon pillars. In structures like this one, wafer bowing and layer cracking are absent, allowing single-crystal integration of different materials onto a silicon substrate, which serves as a platform for many applications, such as multiple-junction solar cells, x-ray and particle detectors, or power electronic devices. See page 1330.

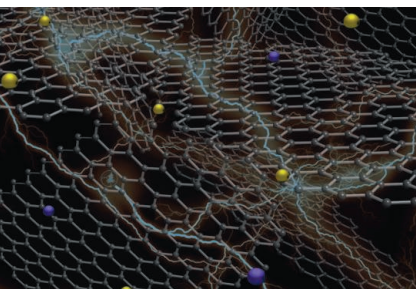
Image: Claudiu V. Falub, Laboratory for Solid State Physics, Swiss Federal Institute of Technology (ETH-Zürich)

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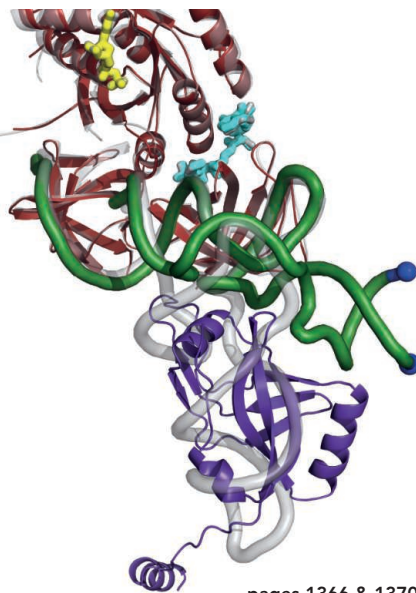
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A Fine-Scale Chimpanzee Genetic Map from Population Sequencing

A. Auton et al.

Chimpanzees show similar genetic recombination rates as humans but differ in the genomic regions involved.

10.1126/science.1216872

Ribosome Profiling Shows That miR-430 Reduces Translation Before Causing mRNA Decay in Zebrafish

A. A. Bazzini et al.

MicroRNAs act to repress their messenger RNA targets first by blocking translation initiation and then through degradation.

10.1126/science.1215704

ESCRT-III Governs the Aurora B–Mediated Abscission Checkpoint Through CHMP4C

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The membrane scission and cytokinesis ESCRT machinery may help to protect against genetic damage.

10.1126/science.1217180

The Coexistence of Superconductivity and Topological Order in the Bi₂Se₃ Thin Films

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10.1126/science.1216466

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Comment on “Widespread RNA and DNA Sequence Differences in the Human Transcriptome”

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Full text at www.sciencemag.org/cgi/content/full/335/6074/1302-c

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M. Li et al.

Full text at www.sciencemag.org/cgi/content/full/335/6074/1302-f

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A study raises hopes that some species may be more resilient than previously thought.

http://scim.ag/Corals_Seas

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http://scim.ag/Penguins_Triumph

To Boldly Go Where No Bee Has Gone

A genetic study of bees suggests that similar genes confer novelty seeking in insects and humans.

<http://scim.ag/Scout-Bees>

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The Signal Transduction Knowledge Environment

13 March issue: <http://scim.ag/ss031312>

RESEARCH ARTICLE: Regulation of the EGF Transcriptional Response by Endocytic Sorting

B. Brankatschk et al.

The transcriptional response to EGFR activation is rapidly initiated after receptor stimulation before degradative sorting occurs.

RESEARCH RESOURCE: Proteome-Wide Discovery of Evolutionary Conserved Sequences in Disordered Regions

A. N. Nguyen Ba et al.

A statistical analysis method can identify short, functionally important linear motifs in disordered regions of proteins.

PERSPECTIVE: From Sulfenylation to Sulfhydration—What a Thiolate Needs to Tolerate

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New techniques have revealed the dynamics of posttranslational modifications triggered by redox signaling that target cysteine residues.

E-LETTERS: Comment and Response on “Ras and Rap GAP Function and GTPase Sequestration in Plexin-Mediated Cell Signaling Mechanisms”

Negishi and Buck discuss the work of Wang *et al.* in the context of other studies of the mechanisms by which plexins transduce signals.

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Integrating Medicine and Science

14 March issue: <http://scim.ag/stm031412>

STATE OF THE ART REVIEW: Treating Human Autoimmunity—Current Practice and Future Prospects

M. D. Rosenblum et al.

Recent advances in understanding immune regulation set the stage for new targeted therapies for autoimmune disease.

RESEARCH ARTICLE: A Model for Personalized in Vivo Analysis of Human Immune Responsiveness

H. Kalscheuer et al.

Personalized humanized mice can model intrinsic defects in human immune disease.

RESEARCH ARTICLE: Data-Driven Prediction of Drug Effects and Interactions

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RESEARCH ARTICLE: Structure-Guided Design of a High-Affinity Platelet Integrin $\alpha_{IIb}\beta_3$ Receptor Antagonist That Disrupts Mg²⁺ Binding to the MIDAS

J. Zhu et al.

A new integrin-directed drug blocks platelet aggregation and may have fewer side effects.

SCIENCE CAREERS

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Data Deluge Drives Demand

E. Wayman

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Tooling Up: Customize Your Training

D. Jensen

The extra skills you need to get attention from industry do not have to cost an arm and a leg.

http://scim.ag/TU_CustomizeTraining

In Person: Career GPS

E. Shkolnik et al.

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<http://scim.ag/InPersonGPS>

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Science Policy News and Analysis

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ADVANCING SCIENCE, SERVING SOCIETY

Pacinian corpuscles are mechanoreceptors tuned to detect high-frequency, low-amplitude, signals. Found in human palm and fingertips, they are useful for discrimination of rough and smooth textures, a sensitivity seemingly amplified by the ridges of fingerprints. Wende *et al.* (p. 1373, published online 16 February) identified a mutation in humans that disrupts this sensitivity to texture, but leaves other facets of touch, such as tactile spatial acuity, intact.

Telling Sandpaper from Satin

Ancient Human Migration

During the past 100,000 years or so, modern humans migrated from Africa into Eurasia, completely replacing existing populations of Neandertals by about 20,000 years ago. This occurred as the climate cooled toward a glacial maximum. Stewart and Stringer (p. 1317) review some of the recent evidence for how this demographic transition occurred. Data from ancient genomes of Neandertals and Denisovans coupled with our improved understanding of the role of refugia in driving evolution during the Ice Ages suggest that such refugia were important in the pace and pattern of change.

Quantum Hall Meets Metamaterial

Controlling and tuning light-matter interaction is crucial for fundamental studies of cavity quantum electrodynamics and for applications in classical and quantum devices. Scalari *et al.* (p. 1323) describe a system comprising an array of metamaterial split-ring resonators and a series of two-dimensional electronic gases (2DEG) formed in GaAs quantum wells. In a magnetic field, the electrons in the 2DEG performed cyclotron orbits and formed Landau levels. Strong coupling was observed between photon and

magnetic cyclotron modes, producing a tunable semiconductor system for studying the light-matter interaction of two-level systems.

Infrared Route to Graphene Electrodes

Electrochemical capacitors can deliver large amounts of power quickly, but have limited energy storage because only the surface regions of electrodes can store charge. Graphene represents an alternative to activated carbon electrodes because of their high conductivity and surface area, but graphene sheets tend to reassociate and lose surface area. El-Kady *et al.* (p. 1326; see the Perspective by Miller) show that graphite oxide sheets can be converted by infrared laser irradiation into porous graphene sheets that are flexible, robust, and highly conductive.

Continental Growth Spurts

The appearance and persistence of continents through geologic time has influenced most processes on Earth, from the evolution of new species to the climate. The relative proportion of newly formed crust compared to reworked, or destroyed, older crust reveals which processes controlled continental growth. Based on the combined analyses of Hf-Pb and O isotopes in zircon minerals, Dhuime *et al.* (p. 1334) measured continuous but variable rates of new crustal production throughout Earth's history. Increased rates of crustal destruction starting around 3 billion years ago coincide with the onset of subduction-drive plate tectonics, slowing down the overall rate of crustal growth.

Which Electron Went Where?

When strong laser fields pull electrons out of atoms or molecules and then send them careening back, the light released on recollision can offer direct insight into local attosecond-scale behavior, or it can be processed into attosecond pulses for probing other samples. When polyatomic molecules are involved, however, it is not always clear which of their electrons are being manipulated by the laser field. Boguslavskiy *et al.* (p. 1336; see the Perspective by Gühr) present a technique for exploring this question. Simultaneous tracking of electrons and fragment molecular ions during strong-field ionization of hydrocarbons revealed the different pathways involved.

Laying It on Thick

The growth of one layered material onto a second lies at the heart of many electronic devices. However, if there is a lattice mismatch between the two materials, strains develop in the overgrowth material leading to bowing and cracking. Falub *et al.* (p. 1330; see the cover) patterned Si substrates into a series of pillars onto which they grew a germanium layer. The germanium initially coated the top of each silicon pillar but then widened as the layer thickened, leading to thick, crack-free germanium films.

Bands That Separate

In organic photovoltaic devices, the charge carriers that form at the interface between donor and acceptor layers—the electrons and holes—form bound states called excitons. Efficient current generation requires some mechanism for their separation and for the movement of free carriers to the electrodes.

Bakulin *et al.* (p. 1340, published online 23 February) studied a process in which the excitons created with an optical pulse were also subjected to infrared pulses. In polymer-blend devices, a three-step process was observed: The bound-state excitons diffused toward the donor-acceptor interface, formed a charge-transfer state, and then dissociated into free carriers.

Invading a Place Like Home

Biological invasions can cause enormous economic problems but they also represent a biological experiment and provide insight into species distributions and range expansion or restriction. Most predictions about when and where species will invade rest on the assumption that invasive species will retain the same climatic niche in the invaded area. But is this assumption valid?

Petitpierre *et al.* (p. 1344) studied a large data set on plant invasions between Eurasia, North America, and Australia and indeed found that fewer than 15% of the studied species occupied more than 10% of invaded distribution outside their native climatic niche, and only one species exhibited >50% climatic niche expansion in its invaded range. Thus, niche shifts are rather rare events in plant invasions.

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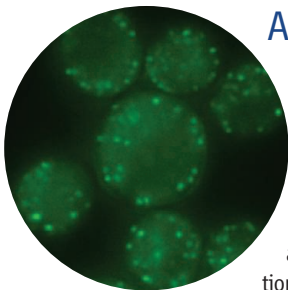
Continued from page 1275

Making Carlactone

Germination of parasitic witchweeds depends on strigolactones, which also regulate plant branching and signal in the context of mycorrhizal symbioses. The biosynthetic pathways that lead to strigolactones are founded in carotenoid biosynthesis, but further steps have been obscure. **Alder *et al.*** (p. 1348) have now identified a biochemical pathway that generates a strigolactone-like compound, carlactone, which shows biological actions similar to those of strigolactone.

Toward Addiction

Addiction can result when substances, such as drugs or alcohol, co-opt the brain's natural reward system. **Shohat-Ophir *et al.*** (p. 1351; see the Perspective by **Zars**) explored this potential in *Drosophila* by examining the relationship between the natural reward stimulated by mating and the unnatural reward offered by ethanol consumption. Males deprived of mating increased consumption of ethanol, and, when permitted to mate following deprivation, their ethanol consumption decreased. At a mechanistic level, mating increased the neurotransmitter neuropeptide F (NPF), while mate deprivation decreased NPF levels.



A Fair COP

During eukaryotic intracellular membrane traffic, how is membrane curvature imparted by the cytoplasmic proteins that form the COPII coat, which mediates vesicle budding from the endoplasmic reticulum? **Čopić *et al.*** (p. 1359, published online 2 February; see the Perspective by **Silvius**) dissected this process by exploiting yeast bypass-of-sec-thirteen (*bst*) mutants, which can survive without the otherwise essential COPII coat protein. These *bst* mutants appear to create a locally altered membrane that is more amenable to deformation by a Sec13-free coat.

A Synaptic Vesicle in Time

Synaptic vesicles move extensively within presynaptic nerve terminals, and their positional features are thought to have an impact on the likelihood and mode of vesicle fusion and transmitter release. **Park *et al.*** (p. 1362, published online 16 February) now provide real-time, three-dimensional tracking of individual synaptic vesicles in living nerve terminals. Single synaptic vesicles were identified within hippocampal neurons right up to the moment of exocytosis.

Ribosome Rescue

Ribosomes stall when they reach the end of defective messenger RNAs (mRNAs). In bacteria, the most-studied ribosomal rescue pathway involves a ribonucleoprotein complex comprising tmRNA (which acts as both transfer RNA and mRNA) and the protein SmpB. In an alternative pathway, some Gram-negative bacteria contain proteins that achieve tmRNA-independent rescue. Now, **Neubauer *et al.*** (p. 1366) present the structure of the *Thermus thermophilus* ribosome bound to a fragment of tmRNA, SmpB, and elongation factor Tu, and **Gagnon *et al.*** (p. 1370) report the structure of the *T. thermophilus* ribosome in complex with an initiator tRNA, a short mRNA fragment, and the rescue factor YaeJ. Though the two rescue systems are very different, both involve a protein tail that binds in the mRNA channel. This orients the rescue apparatus to facilitate switching translation to a different message in the tmRNA system or hydrolysis of peptidyl tRNA by YaeJ.

Diversity in Immune Adversity

Streptococcus pneumoniae commonly colonizes the nasopharynx and has the potential to cause life-threatening infections. Many variants of this pathogen are recognized that have subtly different capsule (an external polysaccharide coat) structures, which prompt distinct immune responses from the host and allow classification of this pathogen into serotypes. A consistent pattern of multiple coexisting serotypes occurs in human populations. **Cobey and Lipsitch** (p. 1376, published online 1 March) probed the mechanisms behind serotype coexistence by developing an ecological model and feeding it data from nasopharyngeal carriage studies.

CREDIT: COPIC ET AL.



Maria Leptin is the director of EMBO, Heidelberg, Germany. E-mail: director@embo.org.

Open Access—Pass the Buck

PEER-REVIEWED SCIENTIFIC PUBLISHING SERVES THE RESEARCH COMMUNITY BY VERIFYING the validity of research results, disseminating the findings, and archiving them in a stable and accessible form. Over the past decade, “open access” has gained momentum as a model for scientific publishing, intended to make results freely accessible to the scientific community and to the public on the Internet. Controversy over public access to research continues to escalate. For example, the dueling proposals recently introduced in the U.S. Congress could have reverberations worldwide: The Federal Research Public Access Act would require articles resulting from research funded by any federal agency to be made publicly available 6 months after publication, whereas the Research Works Act would prohibit such mandates.

Most scientists support the concept of open access. But there is still much debate over the economics and potential consequences of open access among researchers, publishers, universities, funding agencies, and governments. As the director of the European Molecular Biology Organization (EMBO), I discuss this topic extensively with editors of the journals that it publishes, as well as with the community of EMBO researchers. Any transition to open access on a large scale will require a clear understanding of the financial challenges that will be faced. Put simply, publishing costs money, and open access does not mean “for free”—someone must foot the bill. Author fees now range from \$1000 to \$5000 per article, but some journals could require a fee of \$10,000 or more to maintain open access publishing. The cost depends largely on the proportion of submitted articles accepted by a journal. For example, if a journal evaluates 100 articles and publishes all of them at the price of \$1000 each, it will earn \$100,000. However, if it is highly selective, with an acceptance rate of only 15%, the journal still has to evaluate every article, yet it now earns only \$15,000. Thus, in the absence of external support, an open access journal has to be either selective and expensive, or inexpensive but less selective. Currently, highly selective journals running in the open access mode struggle to break even, whereas large-volume, low-selectivity open access publishing generates substantial profit.

Open access was driven in part by anger at the greed, real or perceived, of commercial publishers who were seen to exploit tax-funded research and volunteer academic referees to generate profits. Scientists have embraced open access journals as an appealing alternative.

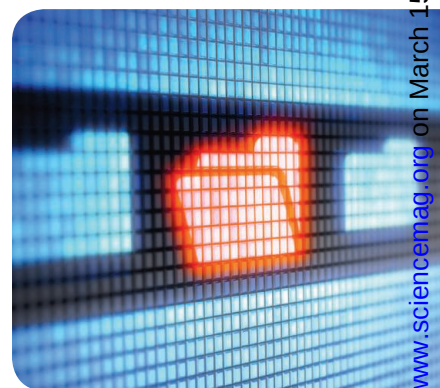
But moving from subscription-based to author-pays economics does not abolish the potential for profit. Furthermore, profit does not necessarily go only into the pockets of the publisher. Professional societies and not-for-profit publishers feed income from their journals back into scientific communities—for example, by providing travel stipends for young researchers. A move to open access means that professional societies will require other funding sources for many of the scientific activities that they finance. Those open access journals that are subsidized face challenges when funding stops. One successful model uses the income from less selective open access journals to finance the highly selective ones.

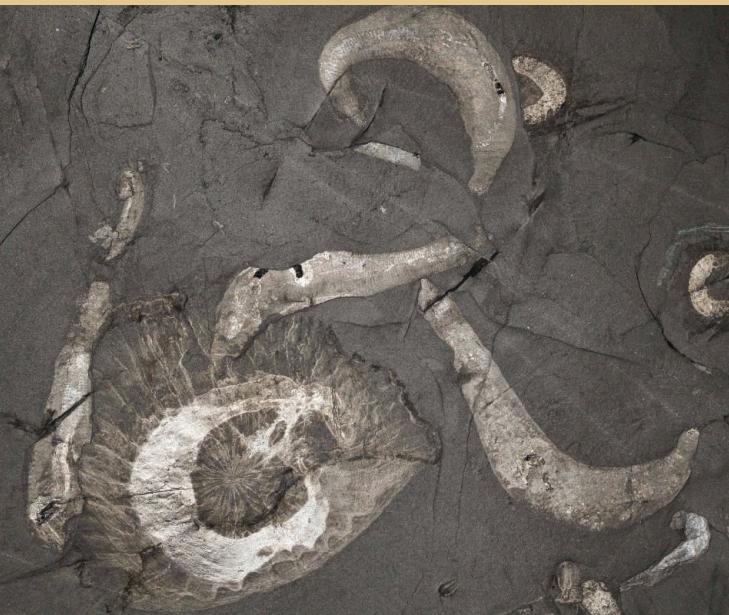
At EMBO, a nonprofit organization with the mission to support top-level research in the life sciences, we publish four journals, two of which are open access. Converting the other two will have to await changes in funding options for open access fees. I believe that an overhaul of the financing of publishing is required. Research funders, intergovernmental agencies, or even governments may need to contemplate direct financing of the costs for open access publishing to minimize the risk of unintended detrimental consequences.

The economics of open access are crucial, but they should not dominate how we think about scientific publishing. We must protect the core principles of scientific publishing no matter what the model: the critical, independent scrutiny of scientific claims and long-term archiving of validated research.

— Maria Leptin

10.1126/science.1220395





PALEONTOLOGY

Preserving the Burgess Shale

The Burgess Shale in British Columbia, Canada, is one of the most famous fossil fields in the world, containing remarkably preserved soft-bodied marine life from just after the Cambrian explosion. In most other fossil locations, these soft, fragile tissues usually degrade in sediments soon after they are deposited. By examining the composition of the overlying sediments and measuring the sulfur isotope composition of minerals surrounding the fossils from the Burgess Shale, the Chengjiang in China, and five other Burgess Shale-type deposits, Gaines *et al.* propose that the unique depositional environment and seawater chemistry composition were the primary factors that facilitated preservation. The seafloor at this time in Earth's history was probably anoxic and also deprived of other oxidants such as sulfate, which inhibited initial bacterial degradation of recently deposited organic remains on the seafloor. Soon after deposition, the fossils were entombed in fine-grained clay sediments and then capped by a thick carbonate cement—a result of increasingly alkaline seawater. The cement further prevented the exchange of oxidants into sediment pore water and allowed the organic matter to remain, eventually transforming into the carbonaceous compressions seen today. — NW

Proc. Natl. Acad. Sci. U.S.A. **109**, 10.1073/pnas.1111784109 (2012).

PHYSICS

Comeback Superconductivity

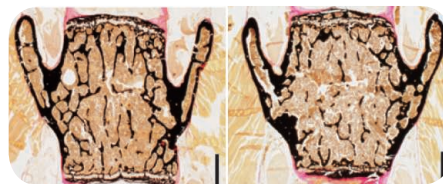
The properties of materials at low temperatures are often tuned by varying chemical composition or pressure. In iron-based superconductors, the transition temperature T_c at which their electrical resistivity plummets to zero exhibits a maximum as a function of both composition and pressure; beyond these optimal settings, T_c monotonically falls to zero. Sun *et al.* report radically different behavior in some representatives of a family described by the formula $A_{1-x}Fe_{2-y}Se_2$ (where $A = K, Rb, \text{ or } Cs$, with possible Tl substitution). In these materials, an increase in pressure beyond the optimal pressure is accompanied by the usual monotonic decrease of T_c down to zero; however, superconductivity unexpectedly and abruptly reappears at even higher pressures and then disappears just as abruptly. Notably, the reemergent T_c is substantially higher than the maximum T_c in the conventional part of the phase diagram (48.7 K for the compound $K_{0.8}Fe_{1.7}Se_2$, approaching the highest T_c for an iron-based superconductor of 55 K). The explanation for this unusual effect awaits further structural and magnetic characterization of the samples under pressure, although preliminary results indicate that the tetragonal crystal structure is preserved throughout. — JS

Nature **483**, 67 (2012).

MEDICINE

Bone of Contention

Vitamin E is a widely used dietary supplement because its antioxidant activity is thought to benefit cardiovascular health. As is true for many supplements, vitamin E's health effects are complex; its role in bone metabolism has been particularly controversial. Two independent studies support the view that the form of vitamin E used in most supplements (α -tocopherol) may adversely affect bone. Fujita *et al.* found



that mice lacking α -tocopherol transfer protein, a model of vitamin E deficiency, had a higher bone mass than controls, because of reduced activity of bone-resorbing cells called osteoclasts. Wild-type mice fed a diet supplemented with α -tocopherol showed a 20% reduction in bone mass as compared to controls; however, the mice were young and undergoing rapid bone growth, so the relevance to effects in adult humans is uncertain. However, data derived from a cross-sectional study of postmenopausal women,

about half taking vitamin E, are broadly consistent with the mouse work. Association between serum levels of α -tocopherol, γ -tocopherol, and markers of bone formation and turnover in the women led Hamidi *et al.* to postulate that vitamin E supplements may negatively affect bone formation. — PAK

Nat. Med. **18**, 10.1038/nm.2659 (2012);

J. Bone Miner. Res. **27**, 10.1002/jbmr.1566 (2012).

BIOMEDICINE

Modeling Ovarian Cancer

Among the four types of epithelial ovarian cancer, which affects about 1.4 % of women, the serous type is found in 70% of ovarian cancer deaths. Studies have reported different origins for ovarian cancer—the ovary or fallopian tube. Kim *et al.* generated a double knockout mouse, eliminating the genes for Dicer, the enzyme that converts pre-microRNAs to mature microRNAs, and Pten, a tumor suppressor, in the reproductive tract, and found that serous carcinomas developed from the fallopian tube and then metastasized elsewhere. All of these mutant mice died within about 6 to 12 months. Single mouse knockouts did not show tumor development in the reproductive system. Abnormal cell proliferation started in the stromal compartment of the fallopian tube rather than the epithelial layer, and the cells underwent a stromal-to-

epithelial transition. The cancers in the double mutant mice were similar phenotypically, histologically, and at the molecular level to those seen in humans. This work supports the idea of a fallopian tube origin of human high-grade serous ovarian carcinoma and provides a model system for future analyses of this deadly cancer in women. — BAP

Proc. Natl. Acad. Sci. U.S.A. **109**, 10.1073/pnas.1201029109 (2012).

CELL BIOLOGY

Seamlessly Hemeless

Hemoproteins are ubiquitous and are required, for example, in respiratory chains, redox reactions, signal transduction, and oxygen transport and sensing. Until now, no eukaryote has been identified that can survive without heme. Kořený *et al.* have discovered a group of evolutionary outliers that defy the omnipresent heme. *Phytomonas serpens* is a plant-parasitic kinetoplastid that has no essential heme-containing macromolecules and a rather odd physiology as a result. The only heme biosynthesis gene *Phytomonas* possesses is ferrochelatase, and it has no respiratory cytochromes, even though it can reoxidize NADH produced during glycolysis, and succinate dehydrogenase still assembles into a functional entity able to reduce ubiquinone. The sugar-rich environment of plant sap allows the organism to bypass the heme requirement for proton pumping, via the non-heme iron of the alternative terminal oxidase. Even in this extreme example, there is enough functional redundancy in the range of cellular processes to allow life to proceed uninterrupted, and *Phytomonas* throws considerable light on potentially undiscovered links and pathways. — CA

Proc. Natl. Acad. Sci. U.S.A. **109**, 10.1073/pnas.1201089109 (2012).

EVOLUTION

Bottleneck Dynamics

Natural or anthropogenic disturbance can reduce the number of individuals in a population very rapidly. When these populations subsequently recover, the individuals in the population will be entirely descended from those that survived the previous disturbance. This process is referred to as a population bottleneck and

is a commonly recognized reason for declines in genetic diversity. Long-term monitoring of a small water vole population on an isolated Scottish island allowed Oliver and Pieltney to document the impacts of a bottleneck driven by the introduction and subsequent removal of sheep. Every individual water vole on the island was identified before, during, and after the sheep-induced population bottleneck, which reduced the population to just five individuals. Monitoring both neutral genetic diversity (in the form of microsatellite DNA) and adaptive diversity (of an MHC allele known to confer resistance to parasites in the population) showed that although genetic diversity, in general, was greatly reduced by the bottleneck, adaptive diversity loss was countered by apparently strong selection for heterozygotes. — SNV

Mol. Biol. Evol. **29**, 10.1093/molbev/mss063 (2012).

ASTROPHYSICS

Magnetic Radio Stars

Magnetars are rapidly spinning neutron stars that are powered by extremely strong magnetic fields. Observationally, they are characterized by x-ray outbursts and by the lack of radio emission. As such, they are widely believed to differ from radio pulsars, which have much weaker magnetic fields and are instead powered by stellar rotation. Over the past 6 years, however, a few magnetars have been observed to show transient, pulsed radio emission after their x-ray outbursts. Rea *et al.* compared the radio emission from these magnetars with that from radio pulsars and concluded that, despite the differences,

radio-emitting magnetars might also be powered by the neutron star's rotational energy, rather than the magnetic energy. The differences in the radio properties of magnetars and radio pulsars might be explained by the differences in the geometry of their magnetic fields. The topology of the magnetic field of magnetars is more complex and is related to their x-ray activity. The knowledge of a magnetar's rotational parameters and x-ray luminosity might thus suffice to predict whether it will emit radio waves. — MJC

Astrophys. J. **748**, L12 (2012).



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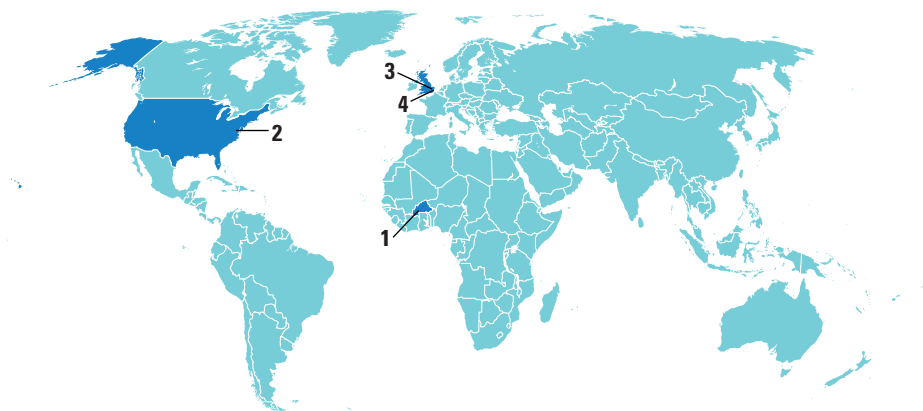
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*The combined member discount when purchasing one of each item at the Apple Store.

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AROUND THE WORLD



Burkina Faso 1

Health on the Air

On 7 March, seven local radio stations in Burkina Faso began broadcasting messages about measures parents can take to treat and prevent deadly disease in their babies and toddlers, hoping to show that such campaigns can be cost-effective public health schemes. The campaign was created by public health experts at the London School of Hygiene and Tropical Medicine (LSHTM) and Development Media International (DMI), a non-governmental organization in London. The 60-second advertisements and 2-hour call-in programs will, for example, encourage pregnant women to get prenatal care, mothers to breastfeed exclusively for 6 months, families to use mosquito nets, and everyone to use clean water and wash their hands.

The project is the first randomized, controlled trial of such mass-media interventions, says DMI's Cathryn Wood. Over



Life-saving message.
Host Kalizeta Soro
broadcasts in northern
Burkina Faso.

the past few months, researchers surveyed residents in 14 regions in Burkina Faso to estimate the current rate of child mortality. Seven regions were randomly assigned to the intervention; the other seven will serve as controls. At the end of the 2.5-year project, researchers will again survey the regions and calculate the campaign's impact on child mortality. A model developed by DMI and LSHTM predicts that an intensive campaign could potentially cut the rate by 15% to 20%.

Washington, D.C. 2

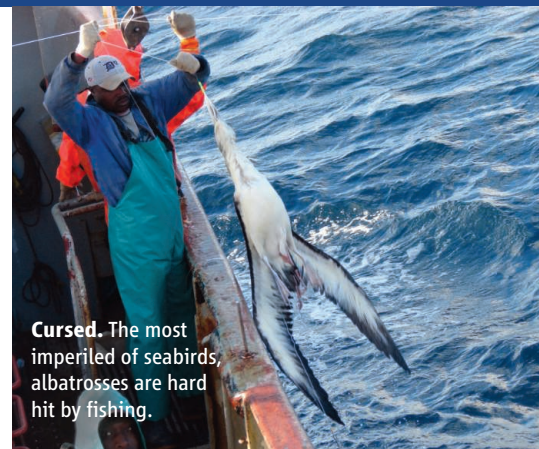
U.S. Senate Approves RESTORE

Ecological and economic restoration efforts along the Gulf of Mexico could be getting a major injection of cash. The U.S. Senate last week approved the RESTORE Act, which would divert 80% of any fines and penalties paid by oil giant BP for the 2010 *Deepwater Horizon* oil spill to restoration activities in five Gulf Coast states. The vote makes it more likely that a majority of the fines—which could total \$5.4 billion to \$21.1 billion—will go to regional activities, including scientific research. That bill could now be taken up by the House of Representatives, which endorsed it last month.

Although many scientists support the measure, some worry that funds are being divided too many ways, and that the act takes a narrow view of what's needed for real restoration. Others fear that state officials will divert funds to projects with questionable ecological benefits, such as beautifying parks or improving beaches.

Still, many advocacy groups celebrated the vote. "We don't have forever," added Senator Barbara Boxer (D-CA), a sponsor. "We need to take care of this today."

<http://scim.ag/Restoreact>



Cursed. The most imperiled of seabirds, albatrosses are hard hit by fishing.

Cambridge, U.K. 3

Seabirds Declining Worldwide, Report Finds

An analysis has found that seabirds are more endangered than other types of birds. Twenty-eight percent of seabird species are threatened, according to a paper published last week in *Bird Conservation International*, while the average for bird families is 12%. The findings "highlight the parlous state of affairs for many of world's seabirds," says ecologist Richard Phillips of the British Antarctic Survey, who was not involved in the research.

Researchers with BirdLife International, a conservation group based in Cambridge, U.K., combed through the International Union for Conservation of Nature's Red List of Threatened Species, tabulating the conservation status, threats, and breeding locations of birds. Of the 346 seabird species, about half have populations that are known or thought to be shrinking.

The main threats facing seabirds include rats and other invasive species that prey on their breeding colonies. In addition, fisheries incidentally kill them and deplete their food. "We have enough knowledge to address these problems today," says lead author John Croxall of BirdLife. "We just need to get on with the job." Approaches include eradicating predators on islands, putting safeguards on fishing gear, and restricting catches.

Harwich, U.K. 4

Stena Lines Won't Ferry Animals for Research

Following a high-pressure campaign from animal rights activists, the last ferry operating company to carry research animals between the United Kingdom and Europe has stopped the service. Academic funders made the change public last week, saying

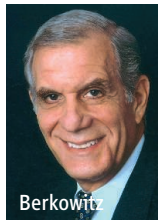
it will complicate animal sharing between international research groups which rely on the transit of live animals when frozen embryos are not practical. Passenger carrier Stena Lines enacted the policy change in January in response to a campaign by the National Anti-Vivisection Alliance. Other ferry companies, including DFDS, also stopped their services in recent months. DFDS spokesperson Gert Jakobsen says managers were sent abusive e-mails and campaigners threatened demonstrations at ports. Carriage of laboratory animals represents a minuscule income stream, Jakobsen says, so DFDS judged it not worth the controversy. Although U.K. government statistics indicate that only three in every 1000 U.K. research animals—most of which are mice—come from abroad, funders, including the Medical Research Council and Wellcome Trust, have warned that constraints on transport of animals could hinder research and lead to more animals being bred. <http://scim.ag/stenaferry>

NEWSMAKERS

Three U.S. Scientists Win 'Arab Nobels'



Varshavsky



Berkowitz



Bussel

A cell biologist and two medical doctors have won three of the King Faisal International Prizes, sometimes called the Arab Nobels. The ceremony was held 6 March in Riyadh, Saudi Arabia.

The Prize for Science went to **Alexander Varshavsky** of the California Institute of Technology for his research into how protein degradation regulates cell functions, which could lead to new therapies for cancer, immune system disorders, and neurodegeneration. Professors **Richard L. Berkowitz** of Columbia University Medical Center and **James Bruce Bussel**, of

Weill Cornell Medical College, both in New York City, shared the Prize for Medicine for their research into the causes and treatment of alloimmune thrombocytopenia, a life-threatening illness in infants.

Computer Scientists Are Joint Waterman Winners

Two computer scientists have been named recipients of the National Science Foundation's (NSF's) Alan T. Waterman Award, which recognizes outstanding researchers under the age of 35 in any science or engineering field. **Scott Aaronson** of the Massachusetts Institute of Technology and **Robert Wood** of Harvard University, both in Cambridge, Massachusetts, will each receive a medal and a \$1 million grant over a 5-year period to continue their research.

Aaronson explores the limitations of quantum computers at MIT's Computer Science and Artificial Intelligence Laboratory and founded the Complexity Zoo



Aaronson



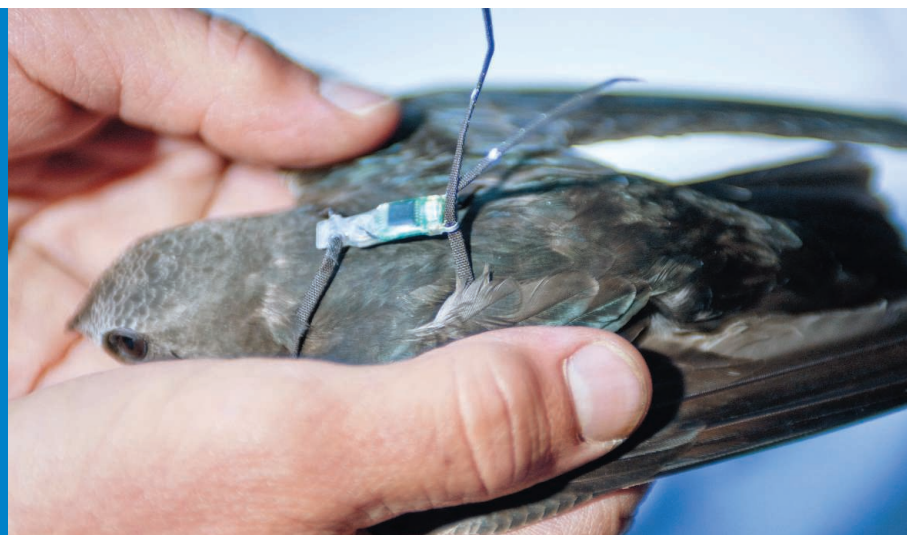
Wood

THEY SAID IT

"Is that telescope going to be strong enough to see the bottom of the financial hole we have dug for it?"

—Representative Jim Sensenbrenner (R-WI) at a 7 March hearing of the House science committee, scolding NASA for cost overruns on the James Webb Space Telescope, whose estimated price tag now stands at \$18.7 billion.

wiki, which catalogs computational problems based on their inherent difficulty. Wood founded the Harvard Microrobotics Lab, where he investigates microfabrication to build biologically inspired miniature robots such as "robobees"—also known as micro air vehicles—for uses as diverse as hazardous environment exploration and crop pollination. >>



Northern Black Swift Mystery Solved

After years of unconfirmed reports and false sightings in Mexico and Central America, scientists now know where the northern black swift (*Cypseloides niger borealis*) goes each winter. These elusive birds wing their way from the misty waterfalls of their breeding range in western North America to wait out the winter in the lowland rainforests of the Brazilian Amazon. Jason Beason of the Rocky Mountain Bird Observatory and his team fitted little backpacks containing geolocator tags to four birds from two breeding colonies in Colorado. The 1.5-gram packs recorded light levels over 1 year; the scientists successfully retrieved data from three of the tags and, using standard astronomical equations, converted those data to latitude and longitude to figure out where these birds disappeared to every fall. The scientists plan to tag birds from other breeding colonies throughout their range to see whether they all head to Brazil or if they go elsewhere.

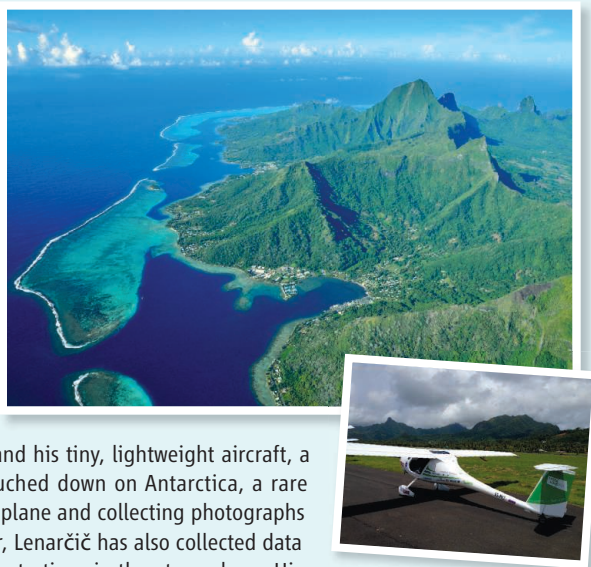
Random Sample

Carbon Sampling Takes Flight

Last month, aerial photographer and biologist Matevž Lenarčič flew a single-seat airplane across 2000 kilometers of airspace between Easter Island and Totegegie Airport in French Polynesia (right). That lone-some leg was one hop on a 3-month journey around the world, during which Lenarčič and his tiny, lightweight aircraft, a Pipistrel Virus (inset), also touched down on Antarctica, a rare solo feat. Between piloting the plane and collecting photographs for an upcoming book on water, Lenarčič has also collected data on black carbon, or soot, concentrations in the atmosphere. His 290-kilogram plane carries a much-lighter-than-normal Aethalometer, designed by aerosol scientist Griša Močnik of Aerosol in Ljubljana, Slovenia, that measures the optical absorption of the atmosphere and converts it to a rough estimate of soot concentration.

Močnik, whose Aethalometers are already used worldwide at ground stations, hopes to learn enough from Lenarčič's flight to build instruments capable of riding piggyback on pleasure flights flown by other aviators: "One could build essentially an ad hoc network of instruments ... in airplanes [whose operators] would voluntarily participate," he says.

Aerosol scientists such as Ryan Spackman of the National Oceanic and Atmospheric Administration in Boulder, Colorado, already use much more sensitive instruments mounted on Gulf Stream jets to collect black carbon data—but such flights are expensive for scientists. Small private aircraft could help fill in a lot of data gaps, particularly at low altitudes near urban areas where soot concentrations tend to be high enough for an Aethalometer to provide very good data, Spackman says. "The first few kilometers [above ground level] are the most interesting."



BY THE NUMBERS

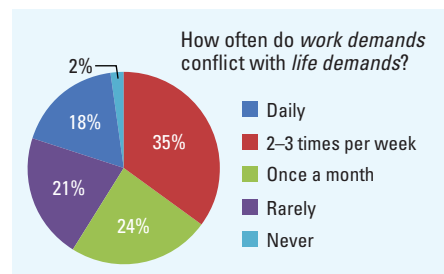
\$5 billion Amount spent on genetic tests in the United States in 2010, according to a UnitedHealth Group study published 12 March. That could go up to \$25 billion within a decade.

30 million tonnes of CO₂ Annual carbon footprint of pumping for China's groundwater irrigation system—similar to the total CO₂ emissions of New Zealand—according to a 14 March study in *Environmental Research Letters*.

FINDINGS

The Global Work-Life Juggle

A majority of scientists in a new global survey say that family responsibilities clash with their professional duties at least twice a week. For U.S. and U.K. scientists it is three out of five, and two-thirds of Canadian scientists face that problem. The data come from a survey of how 4225 active researchers (70% male) are balancing work-



life issues. Preliminary results released last week by the Washington, D.C.-based Association for Women in Science (AWIS) show few gender differences in the attitudes and experiences among younger scientists, says AWIS's Donna Dean, confirming anecdotal evidence from workshops and conferences. The association plans a detailed breakout by country and discipline of the 19-question survey, which had a response rate of 9%.

>>NEWSMAKERS

The 2007 America COMPETES Act authorizes NSF to choose as many as three winners for the annual Waterman award, but this is the first year more than one was named. The \$1 million prize is double the amount of previous years' awards.

Brain Prize Awarded For Work on Hearing

In honor of their contributions to understanding the mechanisms of hearing and deafness, neurogeneticists **Christine Petit** and **Karen Steel** have received the second annual €1 million award from the Grete Lundbeck European Brain Research Foundation, a Danish nonprofit organization.

The foundation praised the two women "for their unique, world-leading contributions to our understanding of the genetic regulation of the development and functioning of the

ear, and for elucidating the causes of many of the hundreds of inherited forms of deafness."

Steel, based at the Wellcome Trust Sanger



Institute in Hinxton, U.K., has used mice to identify genes that disrupt the function of "hair cells" in the inner ear that translate sound waves into neural impulses. She has identified dozens of gene mutations that impair hearing in mice, many of which have also been linked to human hearing loss. Petit, who has appointments at the Institut Pasteur and Collège de France in Paris studies how hair cells contribute to hearing and balance and has identified the cause of at least 10 inherited hearing and balance disorders in humans.



PARTICLE PHYSICS

Key Neutrino Measurement Signals China's Rise

New Year's Day on the traditional lunar calendar is the biggest holiday in China and marks the beginning of a 15-day festival. But there was no break this year for physicists working with China's Daya Bay Reactor Neutrino Experiment 70 kilometers northeast of Hong Kong. Racing to beat competitors around the world to make a key measurement, they worked through the 23 January holiday and surrounding festivities. "Those of us working on the data analysis didn't take time off," says Yifang Wang, co-spokesperson for the Daya Bay collaboration and director of the Institute of High Energy Physics (IHEP) in Beijing. "We were determined to do this."

They succeeded. On 8 March at a seminar at IHEP, the Daya Bay team announced the measurement of a crucial parameter describing the behavior of neutrinos, nearly massless subatomic particles that hardly interact with anything else. The parameter's value suggests that future neutrino experiments, some of which may help explain why the universe contains so much more matter than antimatter, will prove even more fertile than physicists had hoped. "It's not just a parameter; it's a gateway," says Robert Plunkett of Fermi National Accelerator Laboratory (Fermilab) in Batavia, Illinois.

Neutrinos are the chameleons of the subatomic world. The particles come in three different types, or "flavors": electron neu-

trinos, which are born in nuclear reactions; muon neutrinos, which emerge from the decay of particles called pions; and tau neutrinos, which have been generated in particle collisions at accelerator labs. For more than a decade, physicists have known that neutrinos of one type can morph into another type as the particles zip along at near-light speed. For example, electron neutrinos born in the sun change into other flavors so that fewer reach Earth than would be expected otherwise. Similarly, muon neutrinos created when cosmic rays strike the atmosphere change stripes before they reach underground detectors. Those oscillations prove that neutrinos have mass, as they can occur only if the particles have different masses.

In experiments with underground detectors, particle accelerators, and reactors, researchers had measured all but one of the five parameters needed to describe the oscillations theoretically: the two mass differences among the three neutrinos and three abstract "mixing angles" that determine how much one flavor mixes into another. Now, the 250 physicists working with the experiment at the Daya Bay Nuclear Power Plant and two neighboring plants in Shenzhen, China, have measured the last of the three mixing angles, known as θ_{13} .

Researchers measured the flux of electron antineutrinos produced by the six

New dawn. The measurement of a key parameter at the Daya Bay Nuclear Power Plant in China presages an exciting future for neutrino physics.

2.9-gigawatt reactors at the site using three 100-tonne detectors near the reactors and three identical detectors 1.7 kilometers away. They looked for a decrease in the rate at which the antineutrinos reached the distant site, a sign that the particles were oscillating into flavors the detectors could not sense. To measure θ_{13} , researchers had to tease apart two superimposed oscillations: a larger, slower one of the type that accounts for the disappearance of solar neutrinos and reveals a different mixing angle and a smaller, faster one that had not been conclusively observed before. They found that θ_{13} equals 8.8° , give or take about 0.8° .

Daya Bay researchers needed only 55 days' worth of data taking to sew up the measurement. They edged out four other teams racing to make a definitive observation. Last June, physicists working with an accelerator-based neutrino experiment in Japan called T2K, which involves firing a beam of muon neutrinos from a laboratory in Tokai to the subterranean Super-Kamiokande neutrino detector 295 kilometers away, produced a result with a much larger error. Ten days later, researchers working with the MINOS experiment at Fermilab, which fires muon neutrinos 735 kilometers to a detector in the Soudan Mine in northern Minnesota, produced a similar result. In November, researchers with the Double Chooz reactor experiment in France followed suit. The Reactor Experiment for Neutrino Oscillations (RENO) in South Korea is also measuring the angle.

It's no accident that the Daya Bay team leapfrogged the competition, Wang says. By using more detectors with a greater total mass, the researchers could tally neutrinos faster than teams at other reactors could, he says. "One month of our data is comparable to 3 months of RENO's data and 4 months of Double Chooz's," Wang says. Happenstance also played a role, however. The T2K experiment was interrupted on 11 March last year, when the Tohoku earthquake hit Japan. It resumed taking data only in January. Had T2K run as planned, it might have produced an equally precise measurement by now, Wang says.

The fact that θ_{13} turned out to be larger than some scientists had feared also helped to speed up the measurement. And it implies that neutrino physics could be particularly rich

in the decade ahead. For example, researchers in Japan, the United States, and Europe have plans to search for a slight asymmetry between the oscillations of neutrinos and anti-neutrinos, called CP violation, that might help explain why the infant universe grew to contain so much matter and so little antimatter. Those plans would have been for naught if θ_{13} had turned out to be zero. "All these CP-violating effects vanish if θ_{13} is zero," says Paul Langacker, a theorist at the Institute for Advanced Study in Princeton, New Jersey.

Wherever it leads, the result puts Chinese particle physics on the map, researchers say. "This is arguably the most important physics result ever to come out of China," says

Robert McKeown, a Daya Bay team member from the Thomas Jefferson National Accelerator Facility in Newport News, Virginia.

Over the past decade, the Chinese government has ramped up funding for physics and Chinese researchers are returning home from the West, says Wang, who once worked at Stanford University in Palo Alto, California. Still, Chinese researchers must pick their spots, he says. The Daya Bay experiment cost between \$30 million and \$40 million, with China bearing two-thirds of the cost and the rest split among the United States, Taiwan, Russia, and the Czech Republic. "If it cost \$300 million, I don't think we could have afforded it," Wang says.

Over the next 3 to 5 years, Daya Bay researchers will continue to hone the precision of their measurement. After that, they have preliminary plans to build detectors 60 kilometers from the reactors and try to determine the precise ordering of the neutrinos' masses, which should be easier now that θ_{13} is known to be sizable. (Right now, scientists know only the sizes of the differences in the particles' masses.) Of course, rivals such as T2K and the NOvA experiment, under construction at Fermilab, will try to do that first. Given their performance in measuring θ_{13} , don't be surprised if Chinese physicists give the competition a run for their money.

—ADRIAN CHO

DRUG DISCOVERY

New Institute Aims to Help Academics Make Medicines

The pharmaceutical industry is trying to shake itself out of drug-discovery doldrums. One company's latest attempt made its debut this week with the launch of a new non-profit research institute to help academics turn their basic biology insights into drug compounds ready for human tests. The California Institute for Biomedical Research (CalIBR) is being backed by the German pharmaceutical giant Merck, which is committing \$42 million over the first 3 years and possibly as much as \$90 million over the first 7 years.

In return, if CalIBR's collaborations with an outside academic group lead to any drugs ready to move to the clinic, Merck would have the first right to negotiate a deal with the group. If the deal doesn't come together or Merck isn't interested, the academics are free to make a deal with a different pharma partner or spin off their own company.

Peter Schultz, a chemist at The Scripps Research Institute in San Diego, California, and former head of the Genomics Institute of the Novartis Research Foundation, will lead the new institute. Schultz says CalIBR leaders have already begun hiring staff, which he expects to grow to about 150 scientists. The institute is leasing lab space in Torrey Pines, near Scripps and the Salk Institute, but will probably build its own building in the future, Schultz says.

Schultz says the new non-

profit organization is needed to fill a growing gulf between basic biological discoveries made by academic researchers and the work done by pharmaceutical companies to shepherd compounds through human clinical trials to the market. "What we're trying to do is build a bridge that links the novel biology to something that is real," he says. Companies typically put candidate drug compounds through batteries of tests to optimize their effectiveness and limit side effects, but "aca-

dem labs often lack the tools and resources to be able to do that," says Steve Kay, dean of biological sciences at the University of California (UC), San Diego, who is also helping to spearhead the CalIBR effort.

Schultz envisions an academic group approaching CalIBR after it identifies a new disease pathway or drug target, such as a novel protein involved in osteoarthritis. The collaboration would then identify potential compounds that could be honed into a drug to block or bolster the osteoarthritis protein. If successful, the collaboration would then make a deal with a pharma company and split the proceeds 50–50.

"I think it's very forward-thinking," says Kevan Shokat, a chemical biologist at UC San Francisco, who has launched companies of his own in hopes of commercializing basic research discoveries in his lab. If nothing else, the institute will give emerging therapies "more time to percolate" to see whether they can indeed make a difference in patients, Shokat says.

The launch of the new institute comes at a time when pharmaceutical companies around the globe are scrambling in search of a new model for research and development, says Kenneth Kaitin, who heads the Center for the Study of Drug Development at Tufts University in Boston. With the explosion of genomics, proteomics, and other novel science endeavors over the past 2 decades, R&D costs for pharma companies have doubled to nearly \$50 billion annually since 2000, according to the Pharmaceutical Research and Manufacturers of America. Meanwhile, the number of new small-molecule drugs approved for sale every year has leveled off. This combination



"What we're trying to do is build a bridge that links the novel biology to something that is real."

—PETER SCHULTZ,
THE SCRIPPS
RESEARCH INSTITUTE



"One thing I really like about this idea is that at each step in the process we are playing to people's strengths."

—PETER KIM,
MERCK RESEARCH
LABORATORIES

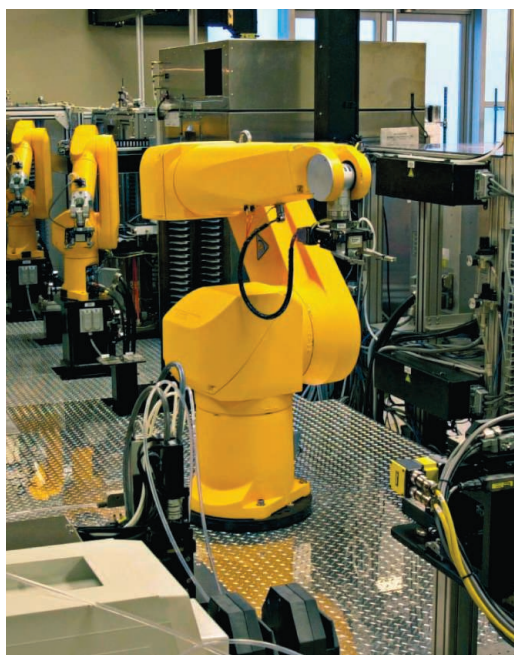
has produced a steady decline in productivity of pharmaceutical R&D and “is essentially a disaster for the industry,” Kaitin says.

In response, pharma companies are becoming more cautious in picking drug compounds to pursue. In many cases they opt to commercialize “me too” drugs similar to those already on the market. Some of the largest companies have also cut back on their internal R&D efforts. That’s left companies with fewer solid drug leads and less ability to develop them in-house, Kaitin says.

One upshot is that pharma companies are increasingly looking to outside sources, including academic labs, for novel drug leads. Yet academic departments are feeling their own crunch with budget squeezes coupled with record-low funding rates on grants from the U.S. National Institutes of Health (NIH), the primary funding agency for biomedical research. “You have two sectors that really need each other right now,” Kaitin says.

Pursuing one popular R&D model, Pfizer, Sanofi, and GlaxoSmithKline have all recently inked deals with universities for first rights to commercialize discoveries from the academic labs. Eli Lilly and Pfizer are also trying an approach in which they let academics run novel drugs they discover through the pharma giants’ high-throughput screening technologies to see whether they are effective and nontoxic.

Schultz says these approaches are all well



Access. A new nonprofit research institute will give academics around the world access to high-throughput drug-discovery robots and other tools.

vania, says his company backed the project because it lets those involved focus on what they do best. “One thing I really like about this idea is that at each step in the process we are playing to people’s strengths,” Kim says. Academics, he explains, excel at discovering novel biological pathways and discovering drug targets. Pharmaceutical companies stand out at pushing well-developed drug leads through human clinical trials. And that would allow CalIBR to specialize in the numerous high-throughput assays needed to prove would-be drug compounds safe, effective, and ready for human trials.

and good. But unlike company alliances with specific universities, CalIBR is free to collaborate with any academic group from around the world. And as a nonprofit, CalIBR will also be in a better position to take on high-risk, high-reward projects that focus on transitioning novel biological insights into the clinic, Schultz says.

Peter Kim, president of Merck Research Laboratories in Upper Gwynedd, Pennsylv-

A major challenge for this new R&D model and most others will be to evaluate whether it’s working, Kaitin says. It normally takes between 8 and 15 years to take a drug all the way from a lead compound to market. So 2 decades might pass before enough compounds have run the gauntlet to show which new R&D model is working best. Nevertheless, Kaitin says, with the drug industry reeling, it’s essential to give this new model and others a try. **—ROBERT F. SERVICE**

ARCHAEOLOGY

Critics Assail Notion That Europeans Settled Americas

Did the first Americans come from Europe instead of Asia? To read some recent news articles, one might think that this long-controversial hypothesis is suddenly about to topple the dominant paradigm, which holds that the earliest Native Americans crossed from Asia to North America over the Bering Strait land bridge at least 15,000 years ago.

The latest media coverage of this paradigm shaker, including major stories in *The Washington Post* and the London-based *Independent*, was sparked by a new book, *Across Atlantic Ice*, by archaeologists Dennis Stanford of the Smithsonian Institution in Washington, D.C., and Bruce Bradley of the University of Exeter in the United Kingdom. Citing similarities in the shape and manufacture of stone tools found on both sides of the Atlantic, Stanford and Bradley argue that at least 20,000 years ago, at the height of the last ice age, people of the Solutrean culture of France and Spain made their way by foot and

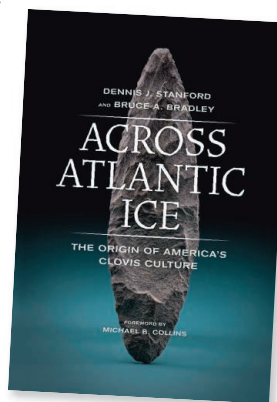
by boat along the edge of Atlantic ice sheets. Eventually, they reached the East Coast of North America, and so were the first to people the New World.

This flies in the face of strong evidence, particularly from genetics, that points to Asian origins for the first Americans. So some researchers are outraged by the notion that the Solutrean hypothesis—which has been a decidedly minority view for decades—is still taken seriously. The idea was “dead on arrival long before” publication of the book, says archaeologist Ted Goebel of Texas A&M University in College Station.

Although few scientists actually support the hypothesis, some still think it

merits additional testing. “We need to keep our minds open,” says archaeologist Tom Dillehay of Vanderbilt University in Nashville, although he, like many others, thinks most of the evidence is still “pointing to Asia.”

When originally proposed in the late 1990s, Stanford and Bradley’s hypothesis linked Solutrean artifacts to those of the 13,000-year-old Clovis culture, the first clearly identified stone-tool-making tradition in the Americas. Since then, additional strong evidence for pre-Clovis tools has come to light (*Science*, 25 March 2011, p. 1512); most experts still think the first Americans hailed from Asia, although earlier than once thought.



Media blitz. A book claiming that the first Americans came from Europe is getting plenty of press.

The Solutrean controversy last flared up between 2004 and 2006, when Stanford and Bradley debated archaeologists Goebel, Lawrence Straus of the University of New Mexico in Albuquerque, and David Meltzer of Southern Methodist University in Dallas, Texas, in the pages of *World Archaeology*. The new book updates the argument with some fresh evidence, such as statistical comparisons of European and American stone tools, including pre-Clovis artifacts, and claims that stone tools found along the East Coast are up to 25,000 years old.

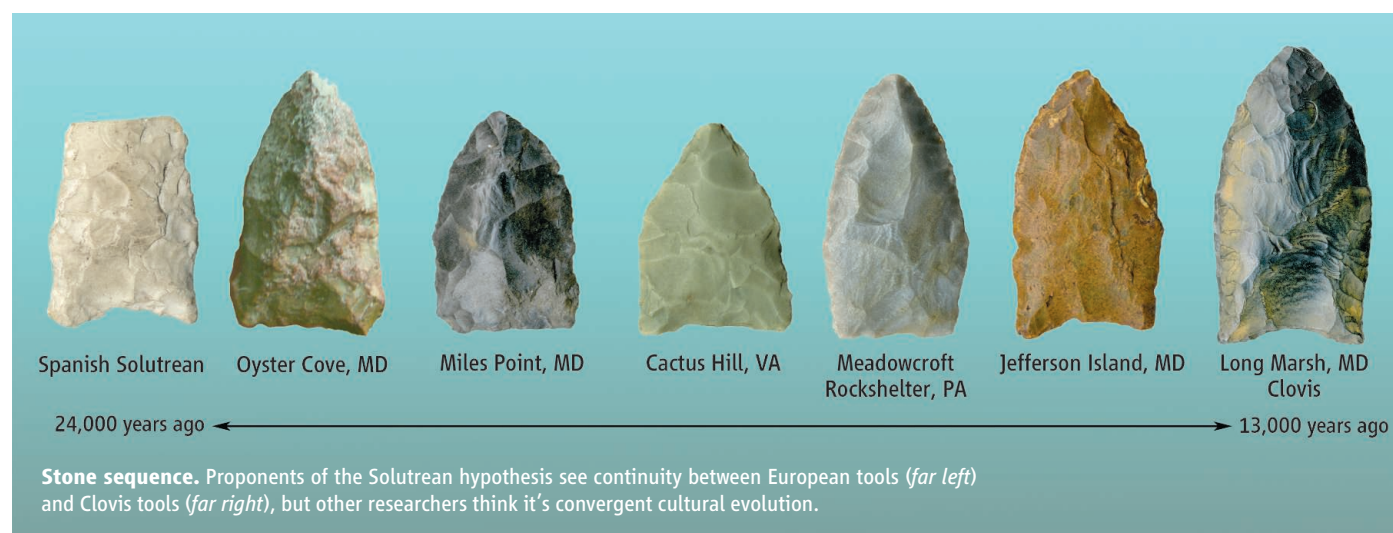
The tool comparisons are a key part of the book, but Meltzer says that the pair continues to “cherry-pick data that supports them,” especially in stressing similarities among Solutrean and American tools, while “glossing over the many differences” (see image).

Miles Point on the Chesapeake Bay in eastern Maryland, geologist Darrin Lowery of the University of Delaware, Newark, dated sediments containing stone tools that Stanford says resemble Solutrean implements to as early as 25,000 years ago. Those dates would make the tools the earliest artifacts in the Americas. Similarly old implements, Stanford and Bradley report in the book, have been found by Lowery and other researchers at several other sites along the East Coast. Archaeologist Michael Waters of Texas A&M, who has seen the Miles Point tools in Stanford’s Smithsonian lab, says that the Maryland location is a “very intriguing site that could be very significant,” but he emphasizes that the work is preliminary and more excavations are needed.

Although confirmation of very early

open Atlantic as Christopher Columbus did.

But the biggest sticking point for the hypothesis, both critics and sympathizers agree, is genetic evidence. “The molecular evidence in support of an Asian origin of Native Americans is overwhelming,” says geneticist Michael Crawford of the University of Kansas, Lawrence. All of the genetic markers that distinguish Native American mitochondrial DNA (mtDNA), as well as their Y chromosomes, can be traced to populations in Asia. None point back to Europe. A similar picture comes from ancient Native American DNA, says geneticist Dennis O’Rourke of the University of Utah in Salt Lake City: mtDNA from North American skeletons as old as 14,000 years still shows the Asian markers (*Science*, 23 September 2011, p. 1692).



Straus, a Solutrean expert, says that the similarities in stone tools can be chalked up to people converging on similar ways to craft artifacts. And archaeologist Michael O’Brien of the University of Missouri, Columbia, says that the pair did not use the statistical methods best suited for exploring historical relationships, rather than mere similarity, between tool types.

This latest skirmish in the Solutrean war is taking place mainly in the media because since 2006, Stanford and Bradley have published little in scientific journals to support their idea. In part, Bradley concedes, that’s because most of their papers “haven’t passed peer review.” Instead, they say, they have concentrated on the book.

But a 2010 paper by Stanford and other researchers, published in *Quaternary Science Reviews*, includes the kind of evidence that the pair think might ultimately turn the tide in the argument. At the site of

stone tools on the East Coast could give the Solutrean hypothesis a boost, opponents say it still has some major strikes against it. For example, Stanford and Bradley have argued that the Solutreans traveled along the edge of an ice sheet that stretched from Spain to Newfoundland, using maritime adaptations—such as seal hunting—that they learned in Europe. But Straus says there is little evidence that the Solutreans exploited the sea to such an extent. Critics also cite a 2008 study published in the *Journal of the North Atlantic* by Kieran Westley, now at the University of Ulster in the United Kingdom, and Justin Dix of the University of Southampton, U.K. Westley and Dix say that the most recent climatic reconstructions of the height of the last ice age, from 24,000 to 18,000 years ago, show that the ice sheet wasn’t continuous across the ocean during most of the year. That would have forced the Solutreans to cross the

But that’s not old enough, Stanford and Bradley counter, especially if the first Americans landed on the East Coast more than 20,000 years ago. In the latest version of their hypothesis, the pair suggest that the Americas might have been peopled from both directions, Europe and Asia; thus Asian genes might have swamped the earlier European ones. “America has always been a great melting pot,” Stanford says.

Ancient DNA expert Eske Willerslev of the University of Copenhagen sides with the critics that no current evidence supports the Solutrean hypothesis. But he also agrees with Stanford and Bradley that researchers need additional older North American DNA, in particular from the much larger and more informative nuclear genome, before the controversy can be resolved. Thus down but not out, the Solutrean hypothesis lives for another day.

—MICHAEL BALTER

INFECTIOUS DISEASE

HIV Prevention and Cure Insights Come From Failure and Success

SEATTLE, WASHINGTON—A dramatic string of successes has buoyed the HIV prevention field over the past 2 years, but a troubling question has lingered: Why did a seemingly simple strategy work in some groups but not others? Last week's meeting* here brought a sobering answer that may guide future prevention efforts. Research on ways to cure HIV infections also received more attention than ever.

One of the most discussed prevention interventions at the meeting, pre-exposure prophylaxis (PrEP), gives anti-HIV drugs to uninfected people. Since July 2010, large clinical trials of PrEP have shown that it works in heterosexual couples with an HIV-infected partner, in men who have sex with men (MSM), and in some single women but not others. At the meeting, an analysis of FEM-PrEP, one of two studies that failed in single women, offered a clear explanation for its disappointing results. (Researchers have not finished assessing the second failure.)

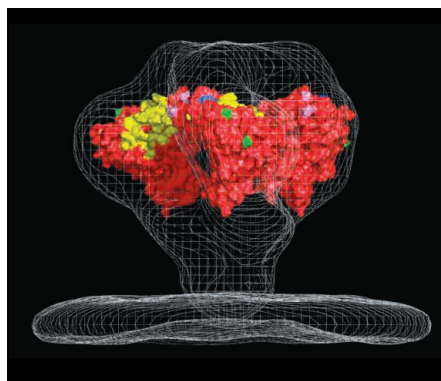
FEM-PrEP enrolled more than 2000 women in South Africa, Kenya, and Tanzania, assigning equal numbers to take either an anti-HIV pill (a combination of tenofovir and FTC) or a placebo each day. Of the 68 women who became infected after the trial started in July 2009, 33 received the drugs, leading researchers to prematurely end the study in April 2011.

Poor adherence to drug regimens often undermines trials, but women reported taking their pills 95% of the time. In an attempt to verify this, researchers required women to return unused pills before they received monthly refills; counts suggested they had taken more than 85% of the drugs (see table). Drug concentrations in the women's blood, however, "told a very different story, despite [our] intense effort to encourage adherence in the trial," said the study's principal investigator, Lut Van Damme of FHI 360, a nonprofit organization in Durham, North Carolina.

The researchers analyzed tenofovir levels in blood samples of treated women taken shortly before and after they became infected. In sharp contrast to the pill counts, less than 26% of these women had a high enough drug concentration to stop HIV. "We do not know at this moment what exactly they were doing with those pills," Van Damme said.

The upside is that PrEP in women likely works if they take the drugs. "The data really line up," said Kenneth Mayer of Brown University, who helped prove PrEP prevents HIV infection in MSM. As a potential solution to the adherence problem, researchers are developing long-lasting drug formulations that require, say, weekly dosing.

Several conference sessions featured



Drug Levels in Blood Explain PrEP Failure Despite Supposed Adherence

	Drug	Placebo
<i>Reported</i> Usually/always took study pill	95%	95%
<i>Reported</i> Easy/very easy to take pills	97%	96%
<i>Measured</i> Pills taken (based on number returned)	86%	89%
<i>Measured</i> Effective drug levels in blood near time of infection	<26%	NA

Mystery solved. FEM-PrEP didn't prevent infections with HIV (its surface protein modeled above) because women didn't take the drugs.

studies that aim to cure HIV—once a far-fetched idea (*Science*, 13 May 2011, p. 784). "It's a real mind change," said Christine Rouzioux, a virologist at Necker Hospital in Paris, who headed one of the sessions. "I fought for years to say the main problem in HIV is the reservoir."

A reservoir of "resting" cells in every HIV-infected person harbors the virus in a dormant state. These resting cells have long lives and fly under the immune system's radar as long as HIV's genes remain hidden inside chromosomes. Even when anti-HIV drugs drive the virus down to undetect-

able levels on standard tests, the resting cells maintain infections. To cure a person, researchers hope to drain reservoirs by waking up resting cells and compelling them to produce HIV, which theoretically will lead the virus to burst cells apart or target them for immune attack.

Two talks at the meeting gave tantalizing hints that drugs can potentially reduce reservoirs. David Margolis of the University of North Carolina, Chapel Hill, gave six HIV-infected people with undetectable levels of virus a marketed cancer drug made by Merck, SAHA, which targets molecules inside of chromosomes that prevent cells from expressing the virus. After one dose of SAHA, all six patients had significant increases in levels of HIV RNA inside of their resting cells, an indicator that latent virus had awoken. Similarly, a team led by Luis Montaner of the Wistar Institute in Philadelphia, Pennsylvania, found that in nine HIV-infected people who received injections with an immune-stimulating drug, pegylated interferon-alpha-2a, reservoirs of HIV-infected cells appeared to have shrunk. "It's very interesting and hypothesis-generating," said Daria Hazuda, a drug developer at Merck Research Laboratories in West Point, Pennsylvania, who collaborates with Margolis.

Yet prodding infected resting cells to produce HIV may not, by itself, reduce reservoirs. Liang Shan, who works in Robert Siliciano's lab at Johns Hopkins University in Baltimore, Maryland, reported dogma-challenging results from a test-tube study with SAHA. As Margolis did in patients, Shan activated HIV in resting cells with SAHA, but in the lab dishes, the virus did not, as expected, burst the cells apart. Adding immune cells from patients on effective anti-HIV treatment, who have weak responses against the virus because they rarely see it, also did not kill the resting cells. "We have to accept that after virus reactivation, the cells which remain at the resting state will probably not be eliminated," Shan said.

Shan suggested that cure strategies must boost the immune response against HIV before activating the latent virus. Sharon Lewin, a virologist at Monash University in Melbourne, Australia, who is studying SAHA in HIV-infected people, said it was a "really beautiful talk and a lovely model." Others were more circumspect, but Shan's findings reminded those seeking an HIV cure of a lesson learned the hard way by many investigators who were surprised by the FEM-PrEP failure: Assume nothing.

—JON COHEN

CREDITS (TOP TO BOTTOM): W. R. SCHIEF ET AL., CURRENT OPINION IN HIV AND AIDS 4, 5 (SEPTEMBER 2009); © 2009 WOLTERS KLUWER HEALTH | LIPPINCOTT WILLIAMS & WILKINS; (TABLE) ADAPTED FROM LUT VAN DAMME/FHI360, CROI 2012

*19th Conference on Retroviruses and Opportunistic Infections (CROI), 5–8 March.

ENVIRONMENTAL POLICY

Something Fishy? NOAA Economics Study Makes Waves

The check is in the mail. It's a phrase with new meaning for Scott Steinback, an economist with the U.S. National Oceanic and Atmospheric Administration (NOAA).

Steinback is mailing checks for up to \$500 to anglers in Massachusetts as part of an unusual study to assess the value of saltwater fisheries. But the research has sparked a fierce public backlash, with some bloggers warning that it hides a government effort to end recreational fishing. One member of Congress has labeled it "irresponsible" and "insane."

NOAA and Massachusetts officials quickly disputed those claims, calling the study a valuable "scientific endeavor." Steinback says the results could help policy-

ing it difficult to quantify its value.

Economists have developed various methods for getting around such problems. "Contingent valuation" surveys, for instance, ask people how much they would pay to preserve a forest or be able to hike in a park, or how much they would want to be compensated if a favorite swimming hole became too polluted to use. But relying on hypothetical choices can lead to flawed valuations, say critics, and some economists say a better approach is to offer people real cash.

That's what was done in a 1979 study published in the *American Journal of Agricultural Economics* that Steinback calls "a real classic." To quantify the value of goose

hunting, researchers offered hunters payments of up to \$200 to give up their licenses for a year. The study was "very intriguing to me," says Steinback, who first read it as a graduate student 2 decades ago. And it became a model when the economist, who is based in Woods Hole, Massachusetts, teamed up with the Massachusetts Division of Marine Fisheries to calculate the value of recreational fishing in salt water.

The study, which began earlier this year, is designed to compare four approaches to valuation. Overall, researchers plan to send surveys to about 1900 holders of 2012 fishing licenses, which cost \$10. They are offering 500 randomly chosen participants from \$15 to \$500 each to give up their licenses, with most of the offers at the lower end of the scale. (The state invalidates the license of anyone who cashes a check.) A second,

matched group is being offered similar but hypothetical payments. Members of a third group are being asked how much they would be willing to pay for a saltwater license. The results will be compared with results from an earlier study that asked anglers a series of questions—such as how far they travel to fish—aimed at indirectly calculating the value of their experience. All told, the study is expected to cost NOAA about \$145,000, with as much as \$75,000

going to the cash payments.

Steinback suspected that the approach might cause a stir, but he says he's been "surprised" by the strength of the blowback. The first surveys led some anglers to worry that the state was aiming to raise fees. A few even feared that the federal government wanted to end recreational fishing by "buying out" anglers. Others questioned the study's cost, especially after a 2 March *Boston Globe* story that was headlined: "In experiment, US offers up to \$500 for fishermen not to fish."

That report apparently prompted Representative Steve Southerland II (R-FL)—a first-term congressman and former funeral parlor operator—to grill NOAA officials about the study during a hearing last week on the agency's 2013 budget request. A member of a subpanel of the Committee on Natural Resources of the House of Representatives, Southerland said he was "blown away" that during a "woeful economic environment," NOAA was "paying recreational fishermen not to fish. This is kind of like driving gas prices up to \$8 or \$9 a gallon, just to see just how much the driving experience means to the American people. This is insane."

No other lawmaker voiced similar complaints, and NOAA officials testifying at the hearing stood their ground. "The entire purpose is [to give] appropriate valuations," which "we are asked to do in many deliberations," noted Eric Schwaab, NOAA's chief of conservation and management.

Outside experts say the study appears worthwhile. "I am not aware of another study like this in the marine environment, ... [and] that is a good thing," says economist James Sanchirico of the University of California, Davis. "It means that the authors have identified a setting where there is a potential for a real contribution to knowledge, which makes the broader impacts of the research greater."

Steinback concedes that his study "is an expensive approach" but says it could save money in the long run by establishing a strong relationship between "hypothetical" and cash-offer surveys. And he says he's moving ahead despite the rhetorical fireworks. The first wave of 125 checks has already gone out, and a second wave is about to be mailed. So far, he reports, about 20 anglers have cashed their checks.

—DAVID MALAKOFF



On the hook. A study of recreational anglers by NOAA economist Scott Steinback, who also enjoys the sport, has drawn barbs from critics.

makers and the courts evaluate the costs and benefits of a range of government policies affecting the environment, including how to calculate monetary damages from human-made environmental disasters, such as oil spills, and natural ones like hurricanes. But economists have long disagreed over the best way to value resources that don't have a clear market price. For example, "you can't just go to the store and buy a recreational fishing experience," Steinback notes, mak-

TOXICOLOGY

Heart-Stopping Revelation About How Chinese Mushroom Kills

BEIJING—Southwest China's hill country has long been stalked by a killer whose victims sometimes drop dead in midsentence. Two years ago, researchers unmasked the likely villain as a mushroom, new to science, which no one had realized is poisonous. Now they have isolated its toxins and propose an astonishing modus operandi.

Over the past few decades, health authorities in China have blamed more than 400 deaths on Yunnan sudden unexplained death. The syndrome exhibits a curious pattern. It strikes almost exclusively during the summer rainy season in highland villages in a narrow altitude band in Yunnan and possibly neighboring provinces. In 2010, an investigation spearheaded by epidemiologist Zeng Guang of the Chinese Center for Disease Control and Prevention (CDC) in Beijing identified the culprit as a small white mushroom (*Science*, 9 July 2010, p. 132), since named *Trogia venenata*.

In the latest work, Liu Jikai, a medicinal chemist at Kunming Institute of Botany, and Chinese CDC colleagues set out to pinpoint the poisons. After months of painstaking effort, Liu says, "we isolated almost every unusual compound" in the mushroom. Three were toxic: two novel unsaturated amino acids* and γ -guanidinobutyric acid, a compound normally found in the brain that induces seizures in lab animals. Unlike virtually all known natural amino acids, which have left-handed chirality, the *Trogia* pair is right-handed. Unsaturated amino acids are known toxins in other poisonous mushrooms, says Kimiko Hashimoto, an organic chemist at Kyoto Pharmaceutical University in Japan who with colleagues isolated a vicious muscle-melting toxin from *Russula subnigricans*, a mushroom found in Asia and North America. Puzzlingly, however, *T. venenata*'s toxins are not particularly potent. "The toxicities of the newly isolated amino acids are rather weak," Hashimoto says.

Also contrary to expectations, the *Trogia* toxins had only a mild effect on the heart. Mice fed an extract from the mushroom died. But the enzyme creatine kinase, a marker for heart attacks and other manifestations of muscle damage, was only slightly elevated in mouse blood—certainly not high enough to explain the toxins' lethality. Drilling deeper

*2R-amino-4S-hydroxy-5-hexynoic acid and 2R-amino-5-hexynoic acid.

into the toxicology data, the team discovered that their mice had extremely low blood sugar. The "profound hypoglycemia" triggered by the mushroom extract "may explain the fatal outcome in humans and experimental animals," Liu and colleagues report in the 5 March issue of *Angewandte Chemie*.



Nastier than they look. *Trogia venenata*'s toxins may starve cells of energy.

Such a mechanism for a toxin is not unprecedented. Ackee, a West African fruit grown widely in the Caribbean, contains hypoglycin, an aptly named toxin that blocks cells from using fatty acids as an energy source. As a result, cells rapidly use up glucose, starving them of energy. After ackee ripens, most hypoglycin in the fruit is converted to benign compounds. But eating the unripe fruit or its seeds can be fatal. Hypoglycin causes Jamaican vomiting sickness, in which victims can suffer seizures, coma, and in rare instances, death.

Similarities in the structures of the mushroom's amino acids and hypoglycin, Liu says, suggest that they "belong to the same class of chemical compounds." *Trogia* and ackee poisonings are not mirror images of each other.

In Yunnan, few people had vomited, says U.S. CDC epidemiologist Robert Fontaine, who is on assignment as a senior adviser to the Chinese CDC. However, he notes, some victims would suffer seizures and lapse into comas before dying. And researchers in Kunming isolated one of *Trogia*'s exotic lefty amino acids from the blood of a previously healthy 27-year-old man who had died suddenly in August 2009, in a village in Yunnan that had experienced repeated sudden-death clusters.

What's happening at the molecular level may be the key to unlocking the riddle of Yunnan sudden unexplained death. Hypoglycin blocks beta oxidation, or the production of ATP from fatty acids, and glucose regeneration, causing hypoglycemia. If *Trogia* toxins were simply suppressing blood sugar, victims might pass out—the brain uses only glucose as an energy source—but they would stand a good chance of recovery. The heart, on the other hand, needs a lot of energy and relies on beta oxidation. "A depressed capacity of heart muscle to use fats as energy" may explain *T. venenata*'s lethality, Fontaine says.

Liu suspects that *Trogia*'s toxins, milquetoast on their own, may have powerful synergistic effects, perhaps abetted by γ -guanidinobutyric acid. The team plans more toxicology tests.

Not everyone buys that explanation. Shi Wu-Xiang, an epidemiologist at Dali University in Yunnan who has studied the syndrome, says that relatives have insisted that some victims never ate the mushroom, while in other cases several family members consumed it but only one or two fell ill. He believes the cause may be minerals in the soil washed into the water supply by summer downpours.

Fontaine says that if hypoglycemia is indeed the mechanism, then *Trogia* toxins should only be fatal if blood sugar falls below a threshold. Eating an insufficient quantity to reach that threshold may have no effect, which would explain why some family members do not succumb. More damning, there hasn't been a single reported case of Yunnan sudden unexplained death since Chinese CDC mounted a campaign in 2009 to warn villagers about *T. venenata*, Fontaine says. "It just stopped," he says. "If something pops up again, then we'll say, 'Whoops, maybe we were wrong and go back and investigate.'" But as a public health threat, he says, the case is closed.

—RICHARD STONE



Who Needs Psychiatrists?

Mental health care is desperately needed throughout the developing world. An Indonesian province is testing an unconventional approach

SIGLI, ACEH PROVINCE, INDONESIA—Nurdin Jalil spent nearly 20 years, almost half his life, in a brick cell built for him by his own family. They forced him to live there because they were afraid of him, as were others in this town near the northern tip of Sumatra. Jalil has schizophrenia, and he attacked another villager with a knife, thinking the other man was a pig. His cell still stands in the family's yard not far from the house. It is about 2 by 3 meters and has a small window and a door, both of which are covered with a grid of metal bars. A small pit in the middle of the concrete floor served as a toilet.

When Laila Kusumawati, a nurse from the nearby district hospital, first encountered Jalil in 2006, he was in bad shape. His hair and nails had grown long, and he was filthy. Kusumawati recalls him lunging at the door to his cell, trying to attack her when she came close. After years of confinement, his foot had atrophied. "He walked, I'm sorry to say this, like a monkey," Kusumawati says. She helped arrange for Jalil to be brought to the hospital, where he got cleaned up and received antipsychotic medi-

cations. Today, he lives inside his family's house and is taking religious studies classes. Clean-shaven and neatly dressed, he says he hopes one day to work again as a fisherman.

Jalil's confinement was born not of cruelty but a lack of alternatives. The nearest psychiatrists work at the crowded mental hospital in Banda Aceh, the provincial capital, a half-day's bus ride over winding mountain roads. For many people in this rural province of fishers and farmers, the roundtrip for a patient accompanied by a family member can cost nearly a month's earnings. Antipsychotic medicine can cost that much again. Many families turn instead to local healers, whose methods include chanting, praying, whipping patients, and poking them with burning sticks to expel bad spirits. When these remedies fail, fam-



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Grass roots. At a village in Aceh, a community mental health nurse (*far left*) leads an educational program on stress.

ilies resort to confinement, or *pasung*, as it is called here. In 2010, at least 200 mentally ill people in Aceh, a province of 4.5 million people, were restrained in chains, stockades, or improvised cells like Jalil's.

This bleak picture may be changing, thanks to efforts started in the wake of the devastating 2004 earthquake and tsunami. Provincial health authorities, aided by foreign advisers, are creating a community mental health program that shifts much of the work traditionally done by psychiatrists to general practitioners, nurses, and even village volunteers. This program is the reason Kusumawati found Jalil, and it has resulted in the treatment of tens of thousands of people with mental illness in Aceh, including more than 100 other *pasung* patients.

Whether nonpsychiatrists can provide adequate psychiatric care is an open question. Only a few careful studies exist. The results are encouraging, but scaling up from controlled clinical trials to the real world is no small challenge. Aceh province is one of several places in the developing world where the concept is being tested on a large scale. If these projects succeed, they could be a model for other developing countries, where mental illness is an enormous, if largely underappreciated, cause of disability and financial hardship. Fewer than a quarter of the people with severe mental illness in low- and middle-income countries receive any treatment, according to the World Health Organization (WHO). Psychiatrists are scarce and often concentrated in overcrowded urban hospitals, where human-rights abuses are all too common. "Up until recently, the story was always a negative one. You know, 'Look at all these terrible things that are happening,'" says Harry Minas, who directs the Centre for International Mental Health at the University of Melbourne in Australia and has helped develop the program in Aceh. "I think we can now start telling some stories about successes."

Online

sciencemag.org

S Slideshow and podcast interview (http://scim.ag/Indo_6074) with author Greg Miller.

After the flood

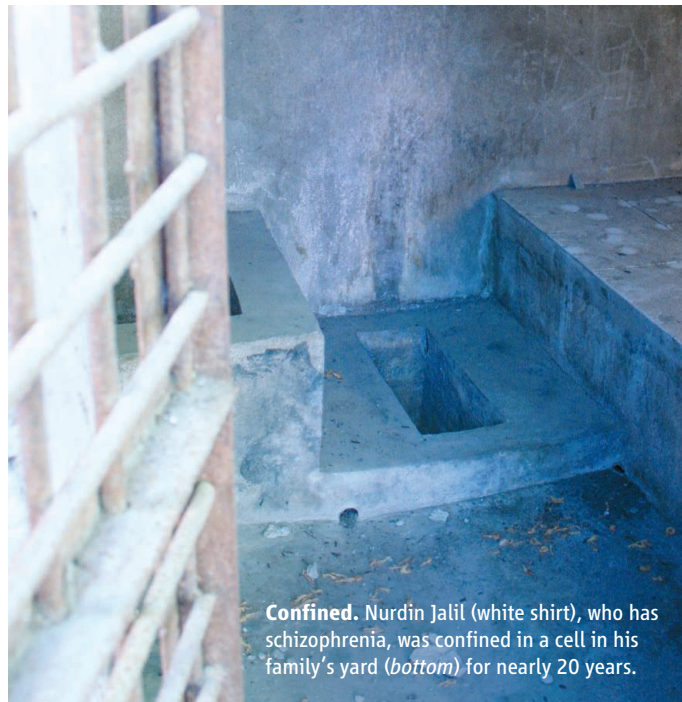
The tsunami, which killed as many as 160,000 people and displaced more than 500,000 in Aceh, was not the province's first taste of tragedy. In the preceding 30 years, armed conflict between Indonesian government soldiers and Acehese separatists claimed at least 15,000 lives. (Some estimates are more than twice that number.) But the deaths are only part of the story, say Byron and Mary-Jo Good, medical anthropologists at Harvard University who have worked in Indonesia since 1996. Sponsored by the International Organization for Migration, the Goods led a survey of psychological trauma in Aceh after the signing of a peace accord in 2005. In three districts heavily affected by the conflict, 78% of people reported witnessing violence, 38% reported having to flee a burning building, and 41% reported having a friend or family member killed. Roughly two-thirds of respondents reported lasting symptoms of depression and anxiety.

Although it was the tsunami that provided the impetus for establishing a mental health system in Aceh, many people here say the conflict has been a greater detriment to the mental health of the population. In this devoutly Muslim region where Shariah police enforce bans on alcohol, gambling, and immodest dress, many people see the tsunami as divine retribution for wicked behavior. The conflict is viewed as more traumatic because it came at the hands of compatriots. "Suffering because of the conflict is unforgivable," says Irwandi Yusuf, the governor of Aceh province. "Suffering because of the tsunami is, let's say, God's make." In part because the Indonesian government barred

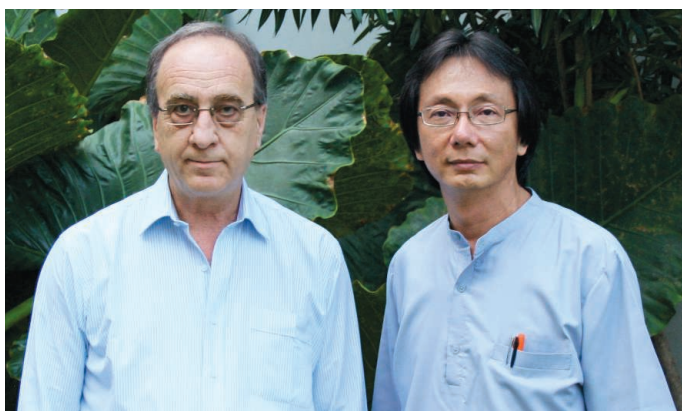
foreigners from Aceh during the conflict, the magnitude of suffering it caused attracted little notice internationally.

The tsunami was a different story. Perhaps because of its timing—televised images of devastation and loss beamed into living rooms around the world just as millions of families in the West were celebrating the Christmas holiday—the disaster triggered a massive outpouring of international concern for the survivors' psychological well-being. Hundreds of foreign aid groups poured into the affected countries with little knowledge of the local culture and, in some cases, outdated ideas about disaster mental health (*Science*, 12 August 2005, p. 1030). Many of these early efforts were ineffective or even damaging, says Benedetto Saraceno, a professor of global health at the New University of Lisbon, who was WHO's director of mental health in Geneva at the time. Despite the initial chaos, Saraceno saw an opportunity in Aceh to create a mental health system that would last beyond the immediate aftermath of the tsunami. He invited Minas to meet him in Jakarta to develop a plan.

Both men had worked in Bosnia during that war and wanted to avoid the mistakes they'd seen there, and saw happening again in Aceh in the



Confined. Nurdin Jalil (white shirt), who has schizophrenia, was confined in a cell in his family's yard (*bottom*) for nearly 20 years.



Advocates for change. Harry Minas (left) and Albert Maramis are proponents of the community mental health project in Aceh.

weeks and months after the tsunami. “In Bosnia, one of the things that was most striking to me was seeing all these trauma centers that had been built and were now empty, with weeds growing up through the floor,” Minas says. “They worked for a while, but they were separate from the government health system and couldn’t be sustained.”

The report Saraceno and Minas prepared for WHO estimated that 100,000 people in Aceh would suffer lasting psychological problems from the tsunami, on top of roughly 60,000 who had preexisting mental illness. But aside from the beleaguered mental hospital in Banda Aceh with its five psychiatrists, mental health care in the province was all but nonexistent.

Momentum for mental health

Aceh was not alone in its neglect of mental health. Until recently, the topic has scarcely been on the radar of health ministries in poor countries. Nor has it factored into major international initiatives like the Gates Foundation’s Grand Challenges in Global Health or the Millennium Development Goals set out by the United Nations. The reason is fairly simple, says Shekhar Saxena, who succeeded Saraceno as WHO’s director of mental health and substance abuse: “They focused on death.” It’s undeniable that infectious diseases such as malaria, AIDS, and tuberculosis kill far more people. But mental, neurological, and substance abuse disorders rival these killers as causes of disability and barriers to economic development (*Science*, 27 January 2006, p. 458). WHO estimates that mental disorders account for a quarter to a third of all years lived with a disability in low- and middle-income countries. “Obviously, death has to be prevented,” Saxena says. “But lives also have to be improved.”

A movement for global mental health seems to be gaining momentum. Last July, an international group of researchers and clinicians published their own set of Grand Challenges in *Nature*, calling for more research and policy changes to improve mental health care. Funding agencies have taken note. Last year the

governments of Canada, the United States, and the United Kingdom announced initiatives that commit millions of dollars for research on global mental health (see table, below).

Much of this research will focus on the feasibility of shifting more mental health care to less specialized, less expensive, and more abundant health workers, says Pamela Collins, who heads an office on global mental health research launched in 2010 by the U.S. National Institute on Mental Health. In wealthy countries, using less specialized workers could reduce health care costs and expand care into underserved areas, she says. In poor countries, it may be the only viable option. In Indonesia, for example, even tripling the number of psychiatrists trained each year would not build their ranks to European levels until well beyond 2050 (see graphic, left panel, p. 1297). That’s why authorities in Aceh have decided to try something different.

It takes a village

At a village meeting hall in Geulumpang Boron, on the east coast of Aceh, a small crowd has gathered to hear a presentation by nurse Fitriassani from the local *puskesmas*, or health clinic. They leave their shoes by the door and roll out straw mats to sit on. The hall has no furniture, but its cement walls and tile floor offer welcome relief from the tropical sun. Bowls of fried plantain strips and bottles of water are passed around. Today’s topic is how to cope with stress, and the group of mostly women seems to be paying close attention, volunteering their own experiences when Fitriassani asks for examples of stressful events they’ve encountered and how they’ve dealt with them.

Community mental health nurses like Fitriassani are the backbone of the program in Aceh. They receive an initial 10 days of training in diagnosis and basic counseling from visiting teams of nurses and psychiatrists from hospitals, universities, and government institutions in Aceh and elsewhere in Indonesia. Nurses who complete the entire program receive an additional 4 months of part-time training. The nurses in turn train cadres, village volunteers, who act as mental health sentries for their community. Nurses visit the villages once or

Investing in Global Mental Health Research

► Integrated Innovations for Global Mental Health

In July 2011, the Canadian government announced \$20 million in funding for 15 to 25 “bold, transformational proposals to tackle the issue of mental health in developing countries.” Awards should be announced by the end of this summer.

► Collaborative Hubs for International Research on Mental Health in Low- and Middle-Income Countries

In September 2011, the U.S. National Institute of Mental Health announced the first awardees in this initiative, and it plans to announce more later this year. The first three hubs, based in Latin America, Africa, and South Asia, will each receive \$2.5 million to \$3.5 million over 5 years for research on how to expand access to mental health care.

► PRogramme for Improving Mental health carE (PRIME)

In May 2011, the U.K. Department for International Development awarded \$9.4 million to a program led by Crick Lund at the University of Cape Town in South Africa that will train general practitioners and nurses to deliver mental health care in Ethiopia, India, South Africa, Uganda, and Nepal, using plans developed by the World Health Organization’s Mental Health Gap Action Programme.

► The Gulbenkian Global Mental Health Platform

In December 2011, the Portuguese-based Calouste Gulbenkian Foundation announced an initiative that will provide \$2.6 million for research in three areas: connections between mental illness and other noncommunicable diseases, strategies for shifting mental health care from hospitals to communities in low- and middle-income countries, and protecting the human rights of people with mental disabilities.

twice a week to check in with their cadres and lead educational programs like the one today on stress. If someone needs more specialized help, they will bring them to the community health center to see a general practitioner trained by the visiting psychiatrists who can prescribe a limited list of psychiatric drugs. For now, the most severe cases still must go to the mental hospital in Banda Aceh.

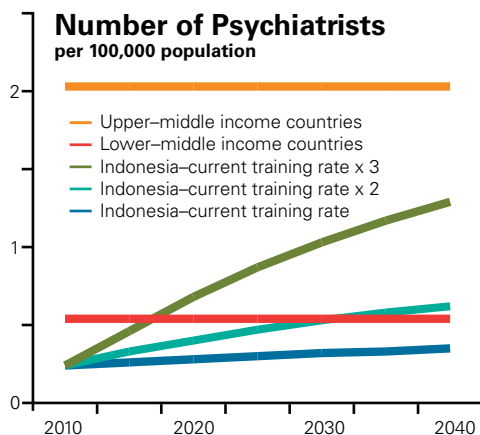
While Fitrissani talks with the women, a smaller number of men sit around the edges. One of them is Mahdi Abdullah, the village leader. He has intense brown eyes and a thick black mustache, and wears two large oval rings on his right hand. It's hard to make a good living here, he says. The primary occupation is growing rice. Aside from that, some men find occasional work doing carpentry or other skilled labor. Before the community mental health program started a few years ago, Abdullah says, mentally ill people and their families had an especially hard time. Other villagers would not only shun the patient but also refuse sometimes to do business with the rest of the family.

The presence of Indonesian troops during the conflict made things worse, Abdullah says. He motions to a man in a green shirt sitting nearby. He is in his early 30s and is rocking slightly, staring vacantly at the floor. Abdullah says the man has been sick for as long as anyone can remember. But his condition deteriorated after he was beaten—

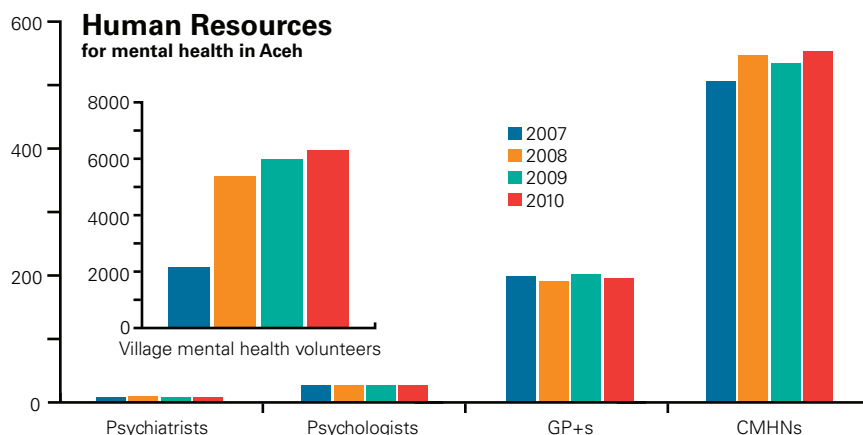
chiatrists are skeptical. Sasanto Wibisono, a professor emeritus and former head of psychiatry at the University of Indonesia in Jakarta, agrees that giving nurses and general practitioners mental health training is a good idea, but he questions having them substitute for psychiatrists. "To overcome a critical situation, it's okay," Wibisono says. But in the long run, he worries that patients treated by nonspecialists won't receive the same quality of care.

Byron Good defends the use of nonpsychiatrists in principle but cautions that it's not a simple fix. "This approach is called for because it seems to all of us the only possible way to provide such care" in poor countries, he says. But so far, he says, there have been "quite few examples of real, evidence-based success that is sustained beyond small, initial pilot projects by highly committed groups." For such a system to work in the long run, doctors and nurses must receive high-quality training and a regular supply of medications, and they should spend a significant portion of their time doing mental health work so that they become proficient at it, Good says. But he adds, "Those conditions are too seldom met."

The project in Aceh does not have funding to assess patient outcomes, but a handful of recent clinical trials with nonpsychiatrists in Chile, Uganda, and other countries have yielded encouraging results.



Task shifting. Even at triple the current training rate for psychiatrists (green line, left panel), it would take decades for Indonesia to catch up to upper-middle-income countries. In Aceh province, an effort is under way to shift some tasks to



more abundant health workers (right panel), including general practitioners and nurses with extra mental health training (GP+s and CMHNs, respectively). Prior to 2005, Aceh had five psychiatrists and no other human resources for mental health.

twice—by Indonesian soldiers because he refused or was unable to answer their questions. He stopped working, which worsened the already tenuous economic situation for his mother and grandmother, with whom he lives.

Abdullah says he's grateful for the mental health program and the changes it has brought to Geulumpang Boron. Villagers now do more to help patients and their families, he says. The man who was beaten by soldiers has received medicine from the nurses (although they say he doesn't always take it), and Abdullah says he now has a job cutting grass to feed another villager's cows.

The doctor won't see you now

Across Aceh, hundreds of general practitioners and nurses have received mental health training since the tsunami, and they have trained thousands of village volunteers (see graphic, right panel, above). Nearly 85% of the community health clinics now have at least some staff with mental health training. In 2010, they saw 28,572 mental health cases, the majority of which previously would have gone untreated, Minas says.

But how effective is the treatment patients receive? Some psy-

The largest trial to date was conducted in Goa, India, by a psychiatrist named Vikram Patel and his colleagues. Patel is a leading figure in the global mental health movement, and he splits his time between the London School of Hygiene and Tropical Medicine and Sangath Centre, a mental health nongovernmental organization he founded in Goa. The researchers screened patients at 12 government-run clinics and 12 private doctors' offices for common mental problems such as depression and anxiety. In a 29-month period, 2796 people who tested positive enrolled in the trial. Half of the patients, selected at random, received routine care from the general practitioners at whichever clinic or doctor's office they'd gone to.

The other half received additional care from young local women trained as counselors. These lay counselors had no health training prior to an 8-week course in which they learned to educate patients about their symptoms and simple things they could do to alleviate them (breathing exercises for anxiety, for example). They also received training in interpersonal psychotherapy, a form of talk therapy that aims to improve mental health by resolving interpersonal problems, such as bereavement or loneliness. For more severe cases, in which the lay counselors' help was not enough, a doctor could prescribe anti-

depressant drugs. Only patients who weren't improving or appeared to be a suicide risk—fewer than 2%—were referred to a psychiatrist.

The lay counselors made the biggest impact at the public clinics. After 6 months, 66% of the patients treated by lay counselors showed improvement in their symptoms, compared with 42% of the patients who'd received routine care at the same public clinics, the researchers reported in 2010 in *The Lancet*. At the private clinics, roughly 65% of patients in both groups improved. (It's possible that the private clinics that volunteered for the study provided better than average care to begin with, Patel says.) Patel is now consulting with the Indian government, which is considering incorporating lay counselors into the national health care system.

In rural Pakistan, psychiatrist Atif Rahman and colleagues enrolled 903 women with maternal depression in a trial. Half of the mothers, chosen at random, received standard care, including visits from "Lady Health Workers," community health workers trained in maternal and child health care. The other half received the same number of visits from Lady Health Workers who had received a short course in basic cognitive behavioral therapy that taught them to listen nonjudgmentally to the mothers' problems and gently guide them toward healthier ways of thinking. A year later, 59% of the mothers in the control group were still depressed, compared with 27% of the mothers treated by the specially trained Lady Health Workers, Rahman and colleagues reported in 2008 in *The Lancet*.

Pakistan has about 350 psychiatrists for a population of 175 million, but there are about 150,000 Lady Health Workers, Rahman says. They earn roughly one-sixth the salary of a psychiatrist, but Rahman says that's not the main point. "I don't see it as [just] a cheaper alternative to specialist care," he says. Instead, he argues, nonspecialists who work in the communities where they live, when properly supervised, can provide more effective care than specialists alone can. If this model gains traction, psychiatrists will find themselves playing a different role than they have in the past, Patel says. They will see fewer patients—only the toughest cases—and spend more time training and managing less specialized health workers.

The findings by Patel, Rahman, and others have been influential in persuading WHO to advocate training for less specialized caregivers as part of its Mental Health Gap Action Programme, launched in 2008, which aims to help developing countries expand mental health care. The organization is helping several countries—Ethiopia, Nigeria, Jordan, and Panama, among others—prepare their primary health systems to deliver mental health care, Saxena says.

The road ahead

Today in Banda Aceh, only a few visible reminders of the disaster remain. A giant power generator barge, untethered from its moorings by the tsunami, remains where the floodwaters carried it, in a residential neighborhood 3 kilometers inland. A monument in a field that abuts the airport road marks a mass grave for tsunami

victims. But houses and roads have been rebuilt, and the cafes and night markets are bustling.

The Norwegian Red Cross has renovated the provincial mental hospital, which flooded in the tsunami, and it's once again a busy place—perhaps too busy. In one ward, scores of men are packed into an open room with spartan beds and bars on the windows. Most mental hospitals in Indonesia are like this: "a big room with too many people," says Albert Maramis, an Indonesian psychiatrist who works for WHO in Jakarta and was stationed in Aceh after the tsunami. During a visit last fall, Maramis was dismayed to see that most patients were locked in their rooms, contrary to international standards for the human rights of patients.

Conditions in Indonesia's mental hospitals need to improve, Maramis and others say, and they have backing from the country's top mental health official, Irmansyah (who, as many Indonesians do, uses one name). But he says his ultimate goal is to establish community-based mental health programs so that fewer patients ever set foot in a mental hospital. Irmansyah applauds the efforts in Aceh, and he says other provinces are interested in replicating them. But he notes that Aceh benefited from the huge influx of foreign aid money after the tsunami. "If you want to copy that model, you have to modify it" to make it more affordable, he says.

Even in Aceh, maintaining momentum won't be easy. In Bireuen, everyone from the village volunteers to the director of the district hospital says they are dedicated to the cause but can only do so much without more training and support. Mursyidah Lathief, a general practitioner at the district hospital, says the mental health training she received from Maramis and his colleagues in Jakarta a few years ago was invaluable. But that experience is a fading memory. "We need regular training like this," she says. The nurses say the same. In Bireuen, several of them work without pay. Community mental health in Aceh clearly has a long way to go.

But if such programs do prove successful, the implications could be far-reaching. Saxena notes that even in wealthy countries, 50% of people with depression don't get treatment. In January, the WHO executive board passed a resolution committing all its member nations—rich and poor alike—to

develop strategies for expanding and improving mental health care. WHO believes nonpsychiatrists can, and must, play a role. "The world is becoming more ready for these changes," Saxena says.

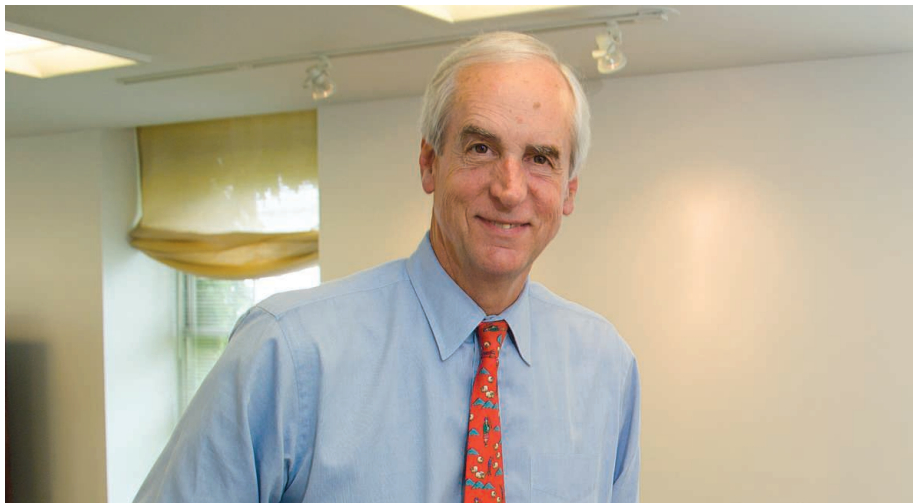
Patel thinks this model could work in other areas of medicine, too, as it already does to some extent in child and maternal health. But he adds that research is needed to identify which interventions can be delivered safely and effectively by nonspecialists. The answers are urgently needed in poor countries where money and medical specialists are scarce, but they're no less relevant in rich countries where health care costs are skyrocketing, he says: "There's a really interesting question here of what can the developed world learn from these experiments born out of necessity?"

In Aceh, the experiment is just beginning.

—GREG MILLER



Too many people. The mental hospital in Banda Aceh, once the only resource for mental health care in the province, remains crowded beyond its capacity.



NEWSMAKER INTERVIEW: HUNTER RAWLINGS III

On Teaching, Tuition, and Talent

U.S. research universities are being asked to improve instruction, hold down costs, and promote economic growth. Are they up to the challenge?

The Association of American Universities (AAU), a group of 61 elite public and private institutions, has traditionally focused on national policies relating to higher education. But under its new president, Hunter Rawlings III, it's also begun to look more closely at what's happening on the campuses of its members. A new initiative aimed at improving the teaching of undergraduate science, technology, engineering, and mathematics (STEM), Rawlings says, represents an issue that is vital to both the nation and the AAU membership.

"We have not been doing a great job of teaching science and engineering, especially to freshmen and sophomores," says Rawlings, a classics professor who took up the reins of AAU last summer and whose résumé includes stints as president of the University of Iowa and Cornell University. "So over a 5-year period, we are going to try and make a difference by disseminating to our members the results of the latest research on teaching and, frankly, advocating for them."

In an interview with *Science* at AAU's Washington, D.C., headquarters, Rawlings predicted that raising the quality of undergraduate instruction will rank high on the to-do list in an upcoming National Academies report on the health of research universities. He also offered some strong opinions on what those institutions need to do to retain their global leadership despite the current stringent fiscal climate. Following is an edited version of that conversation.

—JEFFREY MERVIS

Q: Do you see any likelihood that the steady rise in tuition will slow down?

H.R.: I very much sympathize with universities whose basic instructional budgets are being whacked by 10% or 15% a year by state legislatures. But we need to do a better job of controlling costs. The recession and loss of endowment money has forced a lot of universities to cut costs. But still, tuition is going up, along with room and board and fees. The public and the president are saying they've had enough. The outside pressure is growing to bring it to a halt. I think it's going to slow way down.

Q: The National Institutes of Health is concerned about the aging of its grantees. Is AAU doing anything to address the issue

of older faculty members who don't want to retire?

H.R.: I think it's irresponsible when faculty members stay on in their positions because the law allows them to. At a time when jobs are scarce, I think it's your responsibility to retire and allow the next generation to step forward into academic positions. ... Some departments do a great job of creating the expectation that you're going to retire at age, say, 67, or whatever it happens to be. And the peers make that work. They make it impossible for someone to stay past that point because it's regarded as inappropriate.

Q: There's talk that cuts in state support are turning flagship state universities into quasi-private institutions. What's your view?

H.R.: Well, they aren't going private. They're owned by their states. They'll always be owned by their states. So I think it's bad talk to suggest otherwise.

But it's certainly true that the share of their budgets coming from their states is getting smaller. And that's a shame, because it leads to the perception that education is no longer a public good, which was the understanding we've always had. It's become a private interest, and an economic one at that. And that's not so great. But right now state budgets are also stressed because of the bad economy and the need to spend more on prisons, on K-12 education, on Medicaid, and so on. ...

Fortunately, research universities are remarkably resilient. They have other ways of raising funds. Prisons don't have a whole lot of donors interested in naming cells. But universities have been very successful in raising money, as well as winning grants for research and having industry to invest. So they are doing their best to cope.

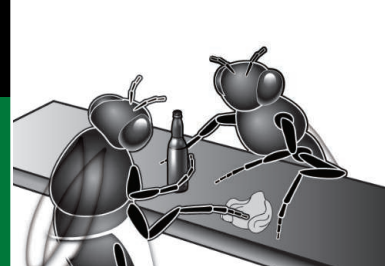
Q: Many universities tout their ability to help stimulate their region's economy. What do junior faculty members need to know about technology transfer, and is there a risk that schools will overpromise?

H.R.: Not to be flippant, but I think it's absolutely fine for a young professor in my field, Greek and Latin, to know absolutely nothing [about commercializing their research]. After all, this is only a portion of what goes on at a research university. But seriously, I think a young researcher in a STEM field has a responsibility, if her work has some possible applications, to be open to someone from the university coming to her and saying, "We think your work has some economic potential. And we'll help you with that."

Q: Are universities doing enough to help graduate students and postdocs who can't find academic jobs?

H.R.: I think we need to do a much better job of making opportunities outside academia accessible to postdocs. We've been focused on cloning ourselves. But nobody is saying, "There are lots of jobs out there for which you are supremely qualified, and we're going to help you."

That being said, you can't plan centrally and federally to produce Ph.D.s at a particular rate and in a particular field. You do your best to shape things in a helpful way, but you're never going to come out evenly, as it were. We need to get smarter at it, but we're never going to solve the problem.



LETTERS

edited by Jennifer Sills

Add Ecology to the Pre-Medical Curriculum

IN THEIR LETTER "COMPETENCIES: A CURE FOR pre-med curriculum" (11 November 2011, p. 760), W. A. Anderson and colleagues endorse a proposed shift in pre-medical education toward core competencies. We believe that the specific competencies proposed by the Association of American Medical Colleges–Howard Hughes Medical Institute report (1) and the corresponding proposed changes to Medical College Admission Tests (2) should include biodiversity and ecological interactions that can influence human health.

A wide variety of species are medically important, both as causes and cures of disease. Approximately 50% of the 100 most-prescribed medicines (3) and 63% of 1073 New Small Molecule Drug Approvals from the Food and Drug Administration between 1980 and 2010 (4) are derived from natural products. Approximately 75% of newly emerging infectious diseases in humans are zoonotic, predominantly from wildlife (5). Many illnesses are induced or exacerbated by environmental factors, including climate and pollution. Understanding the role of species interactions with each other and with the abiotic environment will be crucial to future physicians as they diagnose disease and prescribe medication.

We thus propose an additional core competency for the pre-medical curriculum: "Demonstrate an understanding of taxonomic diversity and fundamental ecological processes and how they relate to human health" (6).

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References

1. Scientific Foundations for Future Physicians, "Report of the AAMC-HHMI Committee" (AAMC, Washington, DC, 2009); www.hhmi.org/grants/pdf/08-209_AAMC-HHMI_report.pdf.
2. AAMC, "5th comprehensive review of the Medical College Admission Test® (MCAT)" (www.aamc.org/download/182662/data/mr5_preliminary_recommendations.pdf).
3. A. S. Bernstein, D. S. Ludwig, *JAMA* **300**, 2297 (2008).

4. D. J. Newman, G. M. Cragg, *J. Nat. Products*, **10.1021/np200906s** (2012).
5. L. H. Taylor, S. M. Latham, M. E. J. Woolhouse, *Philos. Trans. R. Soc. London Ser. B* **356**, 983 (2001).
6. Ecological Society of America, Ecology in the Pre-Medical Curriculum (http://esa.org/education_diversity/ecology_MCAT.html).



Ecology and medicine. Linking ecology, environmental factors, and health in the pre-med curriculum.

IEG's Role in Evaluating Climate Financing

THE INDEPENDENT EVALUATION GROUP (IEG) of the World Bank Group applauds the call by S. D. Donner *et al.* ("Preparing to manage climate change financing," Policy Forum, 18 November 2011, p. 908) for the use of rigorous, empirical evaluation of the impacts of climate finance. With billions of dollars and the planet's climate at stake, and with a quarter of humanity still subsisting on less than \$1.25 per day, it is essential to assess the impacts of interventions on greenhouse gas reduction, poverty reduction, climate resilience, and growth. We need to learn rapidly from successes and failures in these difficult endeavors.

However, the Policy Forum erroneously asserts—without citing data or peer-reviewed evidence—that development banks' internal evaluation groups "rarely find failure, even in the face of strong evidence." In the case of IEG—the largest of the independent evaluation units of the international financial institutions—relevant data is available on our Web site. IEG rated the performance of about three-quarters of World Bank operations that closed in fiscal years 2008 to 2010 as at least moderately satisfactory, whereas the remaining quarter were moderately unsatisfactory at best (1). The Web site also includes the full text of major thematic evaluations, which have been critical where evidence warrants, and which always include recommendations for improved effectiveness.

IEG's recent evaluation of the World Bank Group's climate mitigation investments, in fact, found that those investments failed to adequately invest in feedback and

learn from project experience (inadequacies of monitoring are not limited to World Bank Group projects) (2). The evaluation recommended that the World Bank Group should “measure projects’ economic and environmental impact during execution and after closure and aggregate this information for analysis.” Such information, widely disseminated, would empower evaluators, academics, and stakeholders to undertake their own analyses, supporting the “loose network” of evaluators advocated by Donner *et al.*

Indeed, we believe that such a network would complement IEG. It is important to recognize that all evaluators, internal or external, are potentially subject to bias or conflict of interest. External evaluators, for instance, may depend for funding on the agencies they evaluate. In the case of IEG, there are strong institutional mechanisms to ensure impartiality. IEG reports directly to the World Bank Group’s Board of Executive Directors; Bank Group management has no influence on IEG’s funding and cannot change IEG’s evaluations. This is in accordance with good institutional design for global programs: The governing body of any such program needs an independent source of evaluation as part of its supervision of

management. Thoroughness, impartiality, and insight in evaluation can be supported by a vigorous community of analysts, well-equipped with data.

CAROLINE HEIDER

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References

1. Independent Evaluation Group, “Results and performance of the World Bank Group 2011” (2011); <http://ieg.worldbankgroup.org/content/ieg/en/home/reports/rap2011.html>.
2. Independent Evaluation Group, “Climate change and the World Bank Group: The challenge of low carbon development” (2010); www.worldbank.org/ieg/climatechange/ll/index.html.

Response

HEIDER ASSERTS THE IMPORTANCE OF AVOIDING bias or conflict of interest in evaluating the impacts of climate change financing. We could not agree more. Independent and transparent auditing of the Green Climate Fund (GCF) and other climate change financing is not only critical to minimizing waste, but also to building the public and political will necessary to provide financial support to the developing world. We recognize that internal auditing bodies such as the Independent Evaluation Group (IEG) of the World Bank

Group try to maintain independent governance structures and implement institutional mechanisms aimed at minimizing bias in project evaluation. Unfortunately, there is substantial evidence that historical and ongoing ties between an auditor and the aid institution create the potential for both actual and perceived bias in project evaluation.

Maintaining independence and credibility is a challenge for independent evaluation offices because of shared culture and personnel. There is a revolving door between international development institutions and their internal evaluation groups (1–3). For example, a majority of the current upper management (directors, managers, program leaders, and advisers) at the IEG are former World Bank employees, in some cases for decades. The IEG itself is housed within the World Bank headquarters. It is unlikely that evaluators with long-term ties to the aid institution can conduct investigations free from concern about potential repercussions on a future career in the institution (1). Even if the evaluators are independent, the culture of the institution still affects their outlook and their methods. An external review of the Internal Evaluation Office of the International Monetary Fund (IMF) found that

CORRECTIONS AND CLARIFICATIONS

Review: “The geological record of ocean acidification” by B. Hönisch *et al.* (2 March, p. 1058). The affiliation for author Carles Pelejero was incomplete. The complete affiliation is: “Institució Catalana de Recerca i Estudis Avançats and Department of Marine Biology and Oceanography, Institut de Ciències del Mar, Consejo Superior de Investigaciones Científicas, 08003 Barcelona, Catalonia, Spain.”

News & Analysis: “A tiny window opens into Lake Vostok, while a vast continent awaits” by C. Gramling (17 February, p. 788). At its deepest point, Lake Ellsworth is about 160 meters deep, not 160 kilometers as stated.

Perspectives: “A cold editor makes the adaptation” by M. Öhman (17 February, p. 805). The author’s e-mail address was missing a period between the first and last name; the correct e-mail address is marie.ohman@molbio.su.se. The e-mail has been corrected in the HTML version online.

Reports: “*Cyanophora paradoxa* genome elucidates origin of photosynthesis in algae and plants” by D. C. Price *et al.* (17 February, p. 843). The NCBI Sequence Read Archive (SRA) accession number for the sequence data is incorrect in the Acknowledgments note. The correct number is SRP009206. The number has been corrected in the HTML version online.

TECHNICAL COMMENT ABSTRACTS

Comment on “Widespread RNA and DNA Sequence Differences in the Human Transcriptome”

Claudia L. Kleinman and Jacek Majewski

Li *et al.* (Research Articles, 1 July 2011, p. 53; published online 19 May 2011) reported large numbers of differences between DNA and messenger RNA in human cells, indicating unprecedented levels of RNA editing, and including sequence changes not produced by any of the known RNA editing mechanisms. However, common sources of systematic errors in high-throughput sequencing technology, which were not properly accounted for in this study, explain most of the claimed differences.

Full text at www.sciencemag.org/cgi/content/full/335/6074/1302-c

Comment on “Widespread RNA and DNA Sequence Differences in the Human Transcriptome”

Joseph K. Pickrell, Yoav Gilad, Jonathan K. Pritchard

Li *et al.* (Research Articles, 1 July 2011, p. 53; published online 19 May 2011) reported more than 10,000 mismatches between messenger RNA and DNA sequences from the same individuals, which they attributed to previously unrecognized mechanisms of gene regulation. We found that at least 88% of these sequence mismatches can likely be explained by technical artifacts such as errors in mapping sequencing reads to a reference genome, sequencing errors, and genetic variation.

Full text at www.sciencemag.org/cgi/content/full/335/6074/1302-d

Comment on “Widespread RNA and DNA Sequence Differences in the Human Transcriptome”

Wei Lin, Robert Piskol, Meng How Tan, Jin Billy Li

Li *et al.* (Research Articles, 1 July 2011, p. 53; published online 19 May 2011) reported widespread differences between the RNA and DNA sequences of the same human cells, including all 12 possible mismatch types. Before accepting such a fundamental claim, a deeper analysis of the sequencing data is required to discern true differences between RNA and DNA from potential artifacts.

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Response to Comments on “Widespread RNA and DNA Sequence Differences in the Human Transcriptome”

Mingyao Li, Isabel X. Wang, Vivian G. Cheung

Kleinman and Majewski, Pickrell *et al.*, and Lin *et al.* suggest that mapping and sequencing errors and genetic variants led to false discovery of RNA-DNA sequence differences in our paper. We repeated our analysis using two different sequence alignment methods and carried out additional experiments including whole genome DNA sequencing. The results are consistent with our finding of widespread RNA-DNA sequence differences.

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evaluators were often unable to think outside the box due to the influence of IMF culture and recommended that outsiders be recruited to bring fresh personalities, perspectives, and questioning attitudes (1). It is for these cultural reasons that there have been calls for evaluations of aid institutions to be conducted by people without ties to the institutions (3–5).

In the case of climate change financing, the perception of the trustees and the auditing process could influence whether donor nations meet funding pledges and whether recipient nations trust financing programs. Regardless of recent initiatives to increase aid effectiveness and introduce a culture of learning to aid institutions, the perception of a conflict of interest between the auditor and the aid institution would remain. As Heider notes, this problem would not be solved by delegating evaluation to a single outside entity that could become financially dependent on the institutions it was meant to monitor.

These actual and perceived conflicts of interest can be minimized by engaging a loose, third-party network of audi-

tors through an academic-style peer review system. The internal evaluation divisions at the development banks and aid agencies would still be key players in such a system. For example, if the World Bank becomes the GCF trustee, the IEG could play a more editorial role that includes collecting project data, coordinating the external evaluation process, and reporting results of that process to the GCF Board. This approach would take advantage of the strengths of the IEG while providing the type of transparent auditing necessary to build the political and public confidence in the climate change financing system.

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References

1. K. Lissakers, I. Husain, N. Woods, Report of the External Evaluation of the Independent Evaluation Office (International Monetary Fund, Washington, DC, 2006).
2. C. Weaver, *Rev. Int. Org.* **5**, 365 (2010).
3. A. Lerrick, "Is the World Bank's word good enough?" Testimony before the U.S. Senate Foreign Relations Committee, Hearing on Multilateral Development Banks (U.S. Government Printing Office, Washington, DC, 2006).
4. R. Levine, "Evaluating development aid effectiveness," Testimony before the U.S. Senate Foreign Relations Committee, Hearing on Multilateral Development Banks (U.S. Government Printing Office, Washington, DC, 2006).
5. W. Easterly, "Accountability for multilateral development banks," Testimony before the U.S. Senate Foreign Relations Committee, Hearing on Multilateral Development Banks (U.S. Government Printing Office, Washington, DC, 2006).

Letters

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In This Issue

The modern world can't function without plastics, but the planet's environment may not be able to function with them. Can a new generation of polymer chemists rehabilitate these ubiquitous but environmentally troubling materials?

See full story on page 1382.

Upcoming Features

Innovation in Japan—April 13

Proteomics: Protein Chip Arrays—May 11

Digital Imaging—June 8



Comment on “Widespread RNA and DNA Sequence Differences in the Human Transcriptome”

Claudia L. Kleinman* and Jacek Majewski

Li *et al.* (Research Articles, 1 July 2011, p. 53; published online 19 May 2011) reported large numbers of differences between DNA and messenger RNA in human cells, indicating unprecedented levels of RNA editing, and including sequence changes not produced by any of the known RNA editing mechanisms. However, common sources of systematic errors in high-throughput sequencing technology, which were not properly accounted for in this study, explain most of the claimed differences.

Li *et al.* (1) reported widespread RNA and DNA sequence differences (RDDs) in the human transcriptome, representing all 12 possible residue changes, and suggesting unexpectedly high frequencies and spectra of RNA editing. Because RNA editing and transcriptional noise are known phenomena (2–4), the novelty of this work resides in the extreme extent to which the differences are reported to happen in human cells. However, although high-throughput sequencing (HTS) does provide unprecedented opportunities to study the transcriptome, Li *et al.* did not properly control for a number of technical limitations of HTS and the downstream analysis of the data, resulting in an unacceptably high false-positive rate within this study. In this comment, we revisit the data analyzed by Li *et al.*, highlight the major sources and estimated frequency of errors, and suggest a much more conservative interpretation of the results presented in the original work.

Spurious RNA-DNA differences in HTS data may originate from (i) systematic sequencing artifacts (technology specific), (ii) alignment errors, and (iii) incorrect genotypes resulting from limited coverage of the target regions. The first two types of error will result in specific distributions of presumed polymorphic sites within sequencing reads. Both sequencing and alignment errors most often occur at the termini of reads. In addition, they both tend to occur preferentially on one sequencing strand, whereas for true variants an approximately unbiased distribution can be observed. We analyzed these two biases in the distributions of RNA sequencing calls obtained from the presumed edited sites reported by Li *et al.* as compared with actual confirmed polymorphic DNA sites (Fig. 1, A and B). Based on this analysis, we estimate that false-positive results identified by their presence in only the first or last positions of sequencing reads, along with those that are supported only by unidirectional reads, account for more than half of the entire dataset (Table 1).

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A common alignment error occurs when reads span splice junctions. Short overhangs of a few nucleotides can often be misattributed to an incorrect exon-exon junction. In Fig. 1C, we show

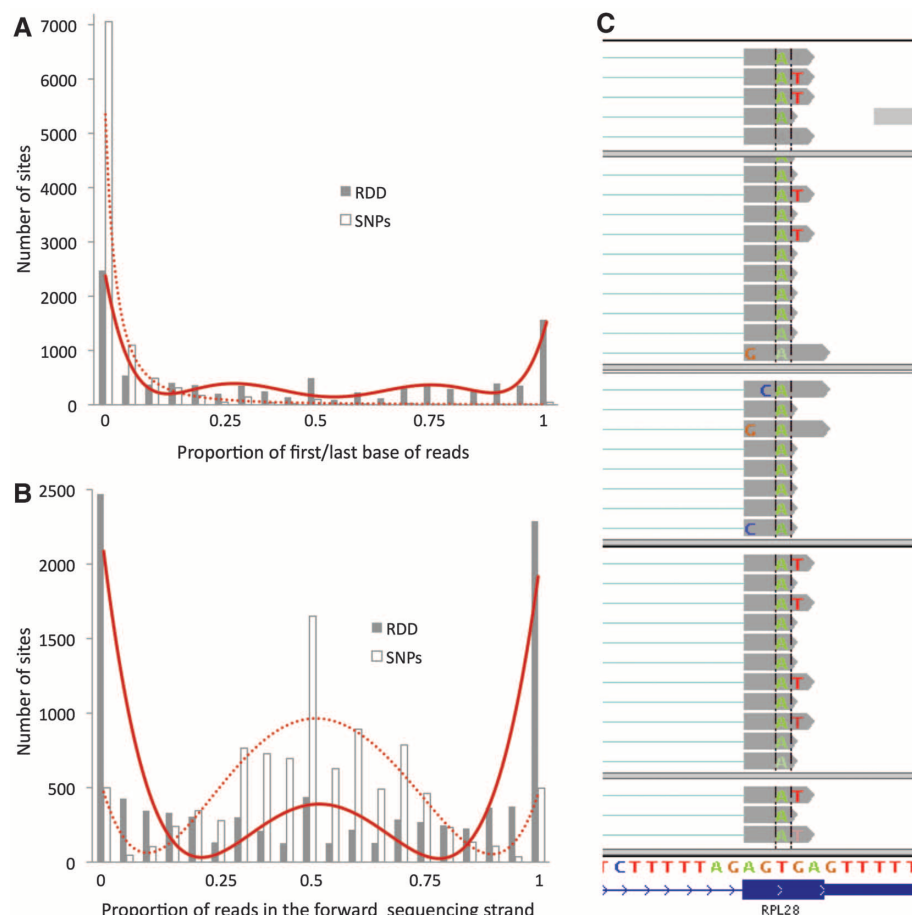


Fig. 1. (A and B) Distributions of RNA sequencing calls observed in RDD sites and in a control data set of dbSNP sites that are polymorphic in the samples under study. False positives are calculated using the tails of the distributions (where the RDD and control distributions intersect), as the number of RDD sites minus the expected number of sites [single-nucleotide polymorphisms (SNPs)]. (See supporting online material for details.) (A) Proportion of reads where the alternative base is found in the first or last base of the sequencing read. Distributions are interpolated using polynomial regression. Solid line, RDD; dashed line, control SNPs. (B) Proportion of reads belonging to the forward sequencing strand. Solid red line, polynomial interpolation of RDD distribution; dashed red line, power law interpolation of control distribution. (C) Screenshots taken from the Integrative Genome Viewer (5), illustrating the read coverage of several samples for the site chr19:60,590,467 in *RPL28*.

one such case in the gene *RPL28*, which was extensively analyzed by the authors as an example of an edited site resulting in a vastly altered protein isoform. This specific case illustrates all the typical features of a false positive alignment and sequencing problem: (i) all the reads align in one direction only; (ii) the variant site is present at the extremity of the read; (iii) it is directly adjacent to another variant; and (iv) it flanks a splice junction and no supporting reads extend past the 5th nucleotide of the exon. It should also be noted that the sequence AGAT, which aligns across the junction, corresponds to the first four bases of the Illumina adapter sequence and is most likely not a part of any expressed sequence but the result of sequencing the adapter. We estimate that more than 700 of the reported RDD sites are due to misalignments close to exon boundaries (Table 1).

Alignment errors are also frequent in repetitive regions and their neighboring sequences. As

Table 1. Major sources of errors, frequency of occurrence in RDD sites, and estimated number of false positives within the RDD data set. (See supporting online material for details.) FPS, false positives.

	Expected*		RDD*		Enrichment†	Estimated FPS
Unidirectional reads‡	1368	13.4%	6658	65.2%	4.9	5289
First/last bases of sequencing reads§	700	6.9%	6184	60.6%	8.8	5484
In exon boundaries	353	3.5%	1100	10.8%	3.1	747
Spanning repetitive regions	327	3.2%	1227	12.0%	3.8	900
In dbSNP 132	87	0.9%	560	5.5%	6.5	473

*Percentages are calculated with respect to the total number of RDD sites: 10,210. †Fold enrichment of RDD sites over expected number of sites. ‡Alternative base is supported by >85% reads going in the same direction. §Sites where the alternative base is supported by >20% first/last bases of sequencing reads.

a solution, the authors use a repeat-masked version of the genome. However, when using short reads, repeat masking with standard parameters is not sufficient because such reads may still map to multiple places. Hence, we identified all regions in the genome allowing multiple mappings of 54 nucleotide reads. RDD sites reported by Li *et al.* are greatly overrepresented in such regions (three-fold over the null expectation), as well as in sequences flanking these and all types of repetitive regions (Table 1).

Finally, very-low-coverage DNA sequencing data was used throughout this analysis, introducing uncertainty in genotype information, particularly for regions that are difficult to sequence. The authors argue that this effect is negligible. However, it should be noted that in the two sam-

ples where the DNA coverage was most complete (~13X), the number of RDD sites detected was lowest and of the same order of magnitude as has been previously reported by others (2). We investigated whether the reported RDD sites are enriched for single-nucleotide variants present in dbSNP132, and found a six-fold enrichment over the null expectation. Given that dbSNP is incomplete, the actual error rate due to missing DNA information is likely to be even higher. As an extreme illustration, four of the six presumed novel exonic coding sites reported by the authors in table 2 have also been reported as polymorphisms in dbSNP.

In fact, for most of the examples cited by the authors throughout the main text, we found a plausible alternative explanation involving a tech-

nical artifact (see table S3). HTS is an exciting new tool for understanding a variety of biological problems and constitutes a very active area of research. With progressively longer sequence reads and improved modeling of sequencing biases in the alignment/assembly steps, it is certainly suited for detecting RDDs. Analyses must, however, take into account both technical and analytical limitations, which the work presented by Li *et al.* does not do properly. We provide evidence that the authors overestimate the frequency of RDDs by an order of magnitude. In view of the above shortcomings, we question some of the boldest findings of this work—i.e., the extent of RNA editing resulting in protein modifications, noncanonical editing types, and variation across individuals.

References

1. M. Li *et al.*, *Science* **333**, 53 (2011).
2. J. B. Li *et al.*, *Science* **324**, 1210 (2009).
3. M. Goldsmith, D. S. Tawfik, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 6197 (2009).
4. M. Meyerovich, G. Mamou, S. Ben-Yehuda, *Proc. Natl. Acad. Sci. U.S.A.* **107**, 11543 (2010).
5. J. T. Robinson *et al.*, *Nat. Biotechnol.* **29**, 24 (2011).

Supporting Online Material

www.sciencemag.org/cgi/content/full/335/6074/1302-c/DC1

Materials and Methods

Tables S1 to S3

References

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Comment on “Widespread RNA and DNA Sequence Differences in the Human Transcriptome”

Joseph K. Pickrell,^{1*} Yoav Gilad,¹ Jonathan K. Pritchard^{1,2}

Li *et al.* (Research Articles, 1 July 2011, p. 53; published online 19 May 2011) reported more than 10,000 mismatches between messenger RNA and DNA sequences from the same individuals, which they attributed to previously unrecognized mechanisms of gene regulation. We found that at least 88% of these sequence mismatches can likely be explained by technical artifacts such as errors in mapping sequencing reads to a reference genome, sequencing errors, and genetic variation.

Li *et al.* (1) sequenced cDNA from lymphoblastoid cell lines derived from 27 individuals whose genomes have been sequenced at low coverage (2) and identified 10,210 sites of mismatches between an individual's mRNA and DNA sequences [RNA-DNA differences (RDDs)]. RDD sites included all possible combinations of sequence mismatches, and the authors validated a subset of these mismatches by additional assays. These observations were interpreted as evidence for novel mechanisms of gene regulation, analogous perhaps to A→I RNA editing (3).

An alternative explanation is that some RDD sites are technical artifacts due to errors in mapping sequencing reads to a reference genome or systematic sequencing errors. To evaluate this possibility, we examined the sequence alignments used to call RDD sites [see supporting online material (SOM)]. Visualizing these alignments revealed a number of anomalies. For example, at the RDD site presented in Fig. 1A, all mismatches to the genome occur at the last base of reads aligned to the negative DNA strand. No such anomalies are seen in alignments around a positive control site (Fig. 1B). The biases in the first example are consistent with several known issues that cause spurious differences between Illumina sequencing reads and a reference genome; these include read-mapping errors between paralogous genomic regions and around insertions and deletions (2, 4), as well as position and strand biases in the error rate of Illumina sequencing (5–7).

We asked whether the patterns seen in Fig. 1A are typical among RDD sites. Indeed, mismatches to the genome at RDD sites are dramatically enriched at the ends of RNA sequencing reads; this contrasts with reads that match the genome at these sites (Fig. 1C). This pattern is evidence that many of the RDD sites are false positives due to mapping or sequencing errors.

To quantify what fraction of RDD sites may be false positives, we used metrics developed for calling single-nucleotide polymorphisms (SNPs) from Illumina sequencing data. In this context, it is known that a search for mismatches between aligned reads and a genome will result in large numbers of false-positive SNPs, many of which can be filtered out based on various criteria (2, 4, 8, 9). We used two criteria based on comparing, at each RDD site, the alignments of RNA sequencing reads that match the genome with the alignments of reads that mismatch the genome—a test for position bias and a test for strand bias (SOM). These tests provide quantitative measures for the intuition that there should be no systematic differences in strand or start position between alignments of reads covering the two alternative genotypes at a site and are similar to tests implemented in SNP-calling packages (4, 9).

The histogram of *P* values for the position bias test for the 7812 RDD sites with at least five reads supporting both bases reveals a clear skew toward low *P* values, indicating pervasive technical artifacts (Fig. 1D). At a *P*-value threshold of 0.01, 87% of these RDD sites fail either the strand bias test or the position bias test (at a *P*-value threshold of 0.05, the corresponding number is 93%). To test the specificity of these filters, we compared the reported RDD sites to a database of known A→I RNA editing sites (10). There are 23 sites in common between the two data sets; of these, 21 (91%) pass both of the filters. This indicates that we are largely only removing false positives.

Genetic variation is another source of false positives; an additional 1% of the putative RDD sites appear instead to be known genetic variants in these individuals (SOM). In total, we estimate that at least 88% (at a *P*-value threshold of 0.01) to 94% (at a *P*-value threshold of 0.05) of the RDD sites are likely false positives. This is probably an underestimate of the true false-positive rate, because some false-positive sites will pass the bias tests by chance and there are additional, unannotated SNPs in the genome.

Given the above results, we reexamined the validation experiments done by Li *et al.* (1). These experiments are of two types. First, at 11 sites, the authors confirmed that the RDD event was absent from genomic DNA but present in cDNA by Sanger sequencing. At 6 of these 11 sites, the event is of the type A→G, and 4 of these 6 are present in a database of known A→I RNA editing sites (10); these are likely true positives. Of the remaining five sites, three fall in a single gene (HLA-DQB2) that is copy number variable in these individuals (11), and one (in the gene DPP7) overlaps a known SNP (at which the reported RDD type matches the known alleles) (2). We suggest that the authors have detected genetic variation rather than RNA-DNA differences at these sites. In sum, these experiments identify two previously unknown sites of A→I RNA editing and provide evidence for a single G→A event.

The second validation experiment involved identifying peptides corresponding to RDD events. In their table 3, Li *et al.* (1) provide 17 examples where both the “DNA form” (the unaltered version) and the “RNA form” (the modified version) of peptides were detected by mass spectrometry. All but one of these sites fail the bias tests described above. We propose that the “RNA forms” of these peptides are in most cases normal forms produced by paralogous genes. Indeed, examination of the “RNA forms” revealed that seven match both the reported protein and additional proteins equally well, and four of the remaining ten match other proteins (in addition to the reported protein) with a single additional mismatch (Table 1 and SOM). It cannot be ruled out that the “RNA forms” of these proteins are instead normal forms caused by genetic variation in their paralogs. An additional possibility is that some “RNA forms” result from sequencing errors in the peptides.

In summary, we estimate that a minimum of 88 to 94% of the RDD sites identified by Li *et al.* (1) are false positives due to mapping errors, sequencing errors, and genetic variation. It is possible that the remainder of RDD sites contain examples of novel mechanisms of gene regulation.

References and Notes

1. M. Li *et al.*, *Science* **333**, 53 (2011).
2. The 1000 Genomes Project Consortium *et al.*, *Nature* **467**, 1061 (2010).
3. B. L. Bass, *Annu. Rev. Biochem.* **71**, 817 (2002).
4. M. A. DePristo *et al.*, *Nat. Genet.* **43**, 491 (2011).
5. K. Nakamura *et al.*, *Nucleic Acids Res.* **39**, e90 (2011).
6. Y. Erlich, P. P. Mitra, M. delaBastide, W. R. McCombie, G. J. Hannon, *Nat. Methods* **5**, 679 (2008).
7. Feacham *et al.*, *BMC Bioinformatics* **12**, 451 (2011).
8. H. Li, J. Ruan, R. Durbin, *Genome Res.* **18**, 1851 (2008).
9. H. Li *et al.*; 1000 Genome Project Data Processing Subgroup, *Bioinformatics* **25**, 2078 (2009).
10. A. Kiran, P. V. Baranov, *Bioinformatics* **26**, 1772 (2010).
11. D. F. Conrad *et al.*; Wellcome Trust Case Control Consortium, *Nature* **464**, 258 (2010).
12. J. B. Li *et al.*, *Science* **324**, 1210 (2009).
13. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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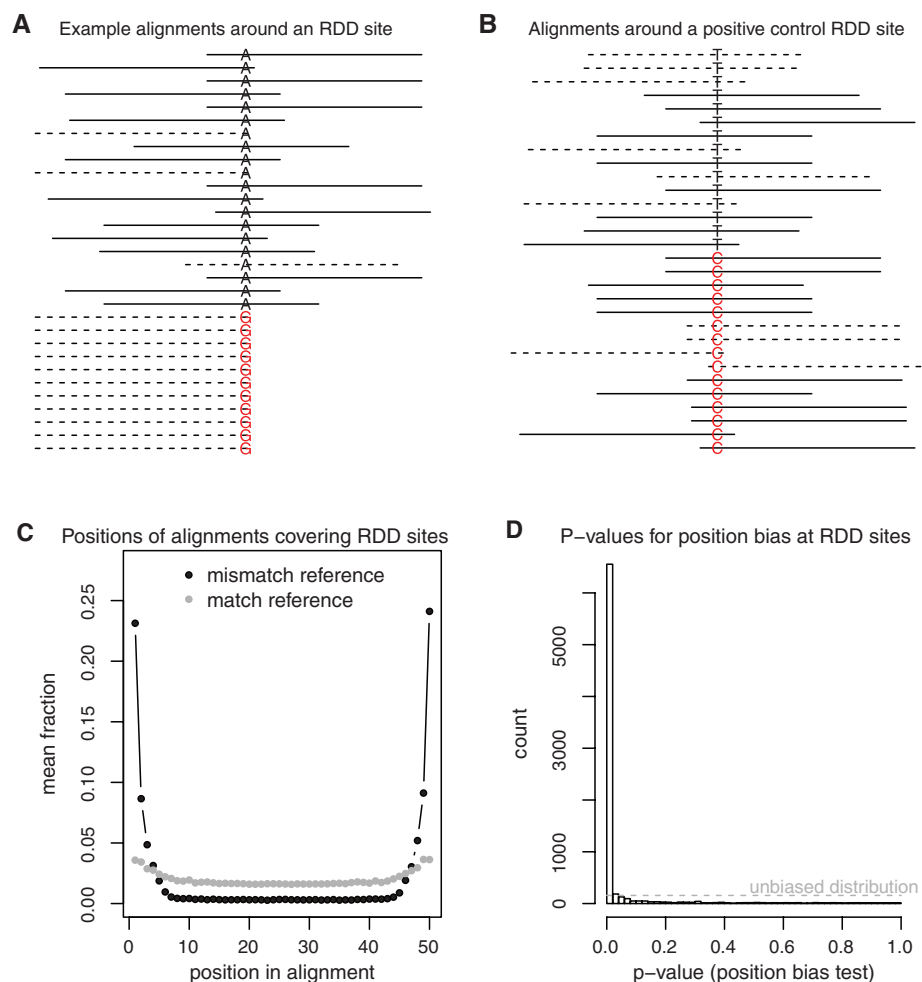


Fig. 1. Identifying false-positive RDD calls. **(A)** RNA-seq read alignments around an RDD call from Li *et al.* (1). Plotted are the positions of read alignments to the genome surrounding the RDD site at chromosome 11, position 105,473,792. The solid lines show sequencing reads aligning to the (+) strand of the genome, and dotted lines are alignments to the (–) strand of the genome. At the center of the plot is the base corresponding to the RDD site; the reference base is in black, the nonreference base is in red, and both are labeled with respect to the (+) DNA strand. Alignments have been organized such that the mismatches to the genome are at the bottom of the figure. For plotting, we randomly sampled 20 alignments that match the genome at the RDD site; all 11 alignments that mismatch the genome are shown. **(B)** Read alignments around a positive control RDD site. Plotted are the positions of read alignments to the genome surrounding the known A→I editing site in AZIN1 (12) (on the forward strand, this site appears as T→C). The format is the same as in (A). For plotting, we randomly sampled 15 alignments that match the genome at the RDD site and 15 alignments that do not match the genome at the site. **(C)** Position biases in alignments around RDD sites. For each RDD site with at least five reads mismatching the genome, we calculated the fraction of reads with the mismatch (or the match) at each position in the alignment of the RNA-seq read to the genome (on the + DNA strand). Plotted is the average of this fraction across all sites, separately for the alignments that match and mismatch the genome. **(D)** Histogram of *P* values for the position bias test. For each RDD site with at least five reads mismatching the genome, we calculated a *P* value for the position bias test (SOM). Plotted is the histogram of these *P* values. If these sites were not consistently biased, the distribution of *P* values would be uniform; this is indicated with the dashed gray line.

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Supporting Online Material

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Materials and Methods

Figs. S1 and S2

References

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Table 1. Characteristics of RDD sites reported in peptides. We reevaluated the peptides presented in table 3 of Li *et al.* (1). Repeated from that table are the gene names, positions and types of RDD sites, and “RNA forms” of protein sequences. We additionally show the numbers of aligned reads that mismatch the genome at each site, and the *P* values from the tests for position bias and strand bias at each site. *P* values in bold are less than 0.01. We used BLAST to search the human

genome for matches to the peptides; given are the names of additional genes [apart from the one reported by Li *et al.* (1)] that match the peptide equally well (because these are the “RNA forms” of the peptides, the best matches have a single mismatching amino acid) and the number of genes with one additional mismatch (for a total of two mismatches) to the peptide. Mismatches are defined as either a substitution or an insertion/deletion of a single amino acid (13).

Protein	Position (hg18)	RDD type	No. RDD reads	“RNA form” peptide sequence	<i>P</i> (dist., strand)	Equally good matches	No. additional close matches
AP2A2	chr11:976858	T→G	3	DLALESMTLASSEFSHEAVK	0.01, 0.59	AP2A1	0
DFNA5	chr7:24705225	T→A	23	VFPQLLCITLNGLCALGR	8 × 10⁻²¹ , 2 × 10⁻⁷	-	0
ENO1	chr1:8848125	T→C	336	EGPELLK	9 × 10⁻⁶⁵ , 8 × 10⁻¹³	C7orf25, ABCF1	>20
ENO3	chr17:4800624	T→G	8	LAQSNWGGMVSHR	0.76, 0.0005	-	2
FABP3	chr1:31618424	T→A	3	MVDAFLGTR	0.007 , 0.07	-	1
FH	chr1:239747217	T→A	37	KEYDTFGELK	1 × 10⁻⁴³ , 2 × 10⁻²⁰	-	0
HMGB1	chr13:29935772	T→A	10	MSSNAFFVQTCR	1 × 10⁻⁹ , 1 × 10⁻⁸	HMGB2	2
NACA	chr12:55392932	G→A	16	DIELVMSQANVSR	3 × 10⁻⁸ , 0.80	-	1
NSF	chr17:42161411	T→C	13	LLDYVPIGPR	2 × 10⁻⁹ , 0.07	-	0
POL2RB	chr4:57567852	T→A	17	IISDGQK	4 × 10⁻¹⁰ , 0.0007	MLKN1, CUL4B	>20
RAD50	chr5:131979610	T→G	9	WRQDNLTLR	1 × 10⁻⁶ , 0.01	-	0
RPL12	chr9:129250509	A→G	518	HSGDITFDEIVNIAR	1 × 10⁻¹⁸⁷ , 7 × 10⁻¹²	-	0
RPL32	chr3:12852658	G→T	356	SAQLAIR	6 × 10⁻⁹⁵ , 8 × 10⁻¹²	RBM46	>20
RPS3AP47*	chr4:152243651	C→A	81	EVQKNDLK	1 × 10⁻⁶² , 1 × 10⁻¹²	-	3
SLC25A17	chr22:39520485	A→G	3	TTHMVLLGIK	0.002 , 0.06	-	0
TUBA1*	chr2:219823379	A→G	33	EDMAALGK	4 × 10⁻⁶ , 6 × 10⁻¹³	CCDC85B, TUBA1B, TUBA1C	9
TUBB2C	chr9:139257297	G→A	9	LHFFMPDFAPLTSR	0.007 , 0.31	TUBB8, TUBB4Q, TUBB6, TUBB2B, TUBB2A, TUBB, TUBB4	1

*The RefSeq name for TUBA1 is TUBA4A, and the RefSeq name for RPS3AP47 is RPS3A.

Comment on “Widespread RNA and DNA Sequence Differences in the Human Transcriptome”

Wei Lin,^{1*} Robert Piskol,^{2*} Meng How Tan,² Jin Billy Li^{2†}

Li *et al.* (Research Articles, 1 July 2011, p. 53; published online 19 May 2011) reported widespread differences between the RNA and DNA sequences of the same human cells, including all 12 possible mismatch types. Before accepting such a fundamental claim, a deeper analysis of the sequencing data is required to discern true differences between RNA and DNA from potential artifacts.

By comparing RNA and DNA sequences generated by high-throughput sequencing of human B cells, Li *et al.* (1) reported finding 10,210 RNA-DNA differences (RDDs) of all 12 possible mismatch categories. Before this study, only two types of RDDs, known as RNA editing, were known to exist in humans, both of which are catalyzed by deaminases (2, 3). One converts adenine to inosine (recognized as guanine); the other converts cytosine to uracil. Li *et al.*'s finding, if confirmed, would add tremendous informational complexity to the transcriptome. However, after examining their data, we discovered several issues that may lead to an exceedingly high rate of false-positive calls of RDDs.

Accurately mapping the short high-throughput sequencing reads is critical for calling RDDs to ensure that RNA- and DNA-derived sequences originate from the same genomic locus. A read derived from one genomic locus can be incorrectly mapped to another paralogous region that arose through genomic duplication. Slight differences between duplicated sequences can be mistaken for RDDs. To estimate the fraction of RDDs that may be miscalled due to duplications, we extracted the flanking sequences (25 bases on each side) of the 10,210 RDDs identified in (1) and aligned them against the human genome with BLAT (4). A total of 1284 sites (~13%) mapped to two or more regions in the genome with >90% identity (Fig. 1). Although Li *et al.* removed all of the segmental duplications (≥ 1 kb) and repetitive regions (1), a large number of known shorter duplications were not removed as they are not considered to be repetitive. In addition, Li *et al.* mapped reads to the transcriptome rather than the genome. Their reference transcriptome (Gencode; www.gencodegenes.org) is potentially missing some paralogous genes, which could lead to erroneous RDD calls.

Even when a read is mapped to the correct genomic locus, that read's alignment with the genome can contain differences primarily for two reasons. First, when a read spans across the splicing junction of an alternatively spliced gene, a misalignment can be easily introduced due to ambiguous mapping of the read end to one of two (or more) possible exons. This is especially true when reads are being mapped to the transcriptome that contains a possibly incomplete annotation of isoforms. Second, the 5' read end, derived from either end of a double-stranded cDNA fragment, may have a higher error rate due to mispriming events introduced by the random hexamers used in the RNA sequencing protocol. Because the alignment tool used in (1), Bowtie, performs semi-global alignment that forces the read ends to be

aligned even when there is a mismatch (5), the two issues above tend to concentrate mismatches to the ends of the reads.

To investigate this further, we took all reads that carry the variant bases at the RDDs and asked where the variants occur along the reads. Consistent with our hypotheses above, there is a clear enrichment of variants at the read ends, particularly at the 5' end (Fig. 2A). However, one could argue that the observed 5' read end bias is precisely due to the presence of RDDs, whereby the first-strand cDNA synthesis may be prematurely terminated immediately after the RDD site is transcribed due to reverse transcriptase incompatibilities with a modified base. To test this possibility, we annotated the originating RNA strand of each read to separate the sequencing reads into two groups: one derived from the 5' end of the first-strand cDNA, and the other from the 5' end of the second-strand cDNA. If the premature termination hypothesis were true, we would expect RDDs to be enriched in the 5' end of the second-strand cDNA. In fact, we found that the opposite is true, where the variants are strongly favored at the 5' ends of the first-strand cDNA (Fig. 2, B and C). We speculate that this phenomenon is primarily rooted in the RNA sequencing protocol. The 5'-end mismatch may be introduced by the random hexamer during the first- and second-strand syntheses. However, the DNA polymerase I used in the second-strand synthesis has 5'→3' exonuclease activity that can remove 5'-end mismatches from the second-strand cDNA. Based on these observations, we trimmed the ends (positions 1, 2, 48, 49, and 50) of all RNA sequencing

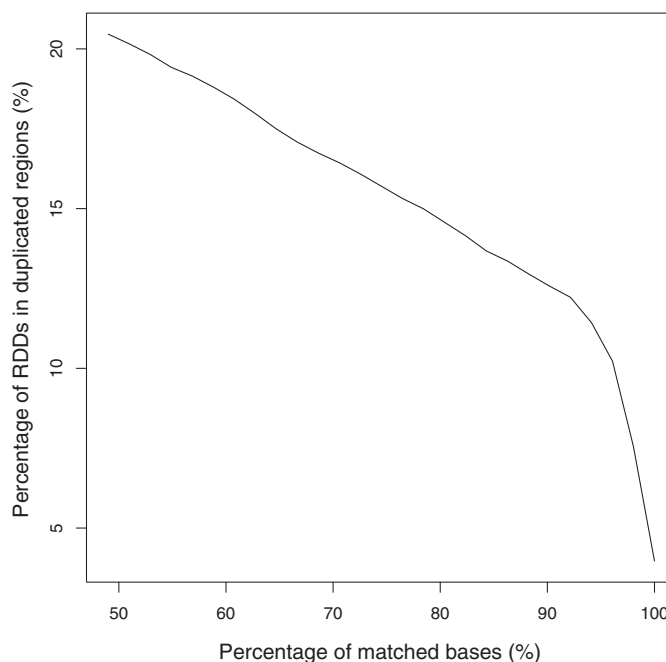


Fig. 1. Percentage of RDDs in duplicated regions. We extracted regions of 25 bp to each side of all RDDs. For each region, the number of hits to the reference genome and the percentage of matched nucleotides was determined. An RDD was considered to be in a duplicated region if more than one hit to the genome was found.

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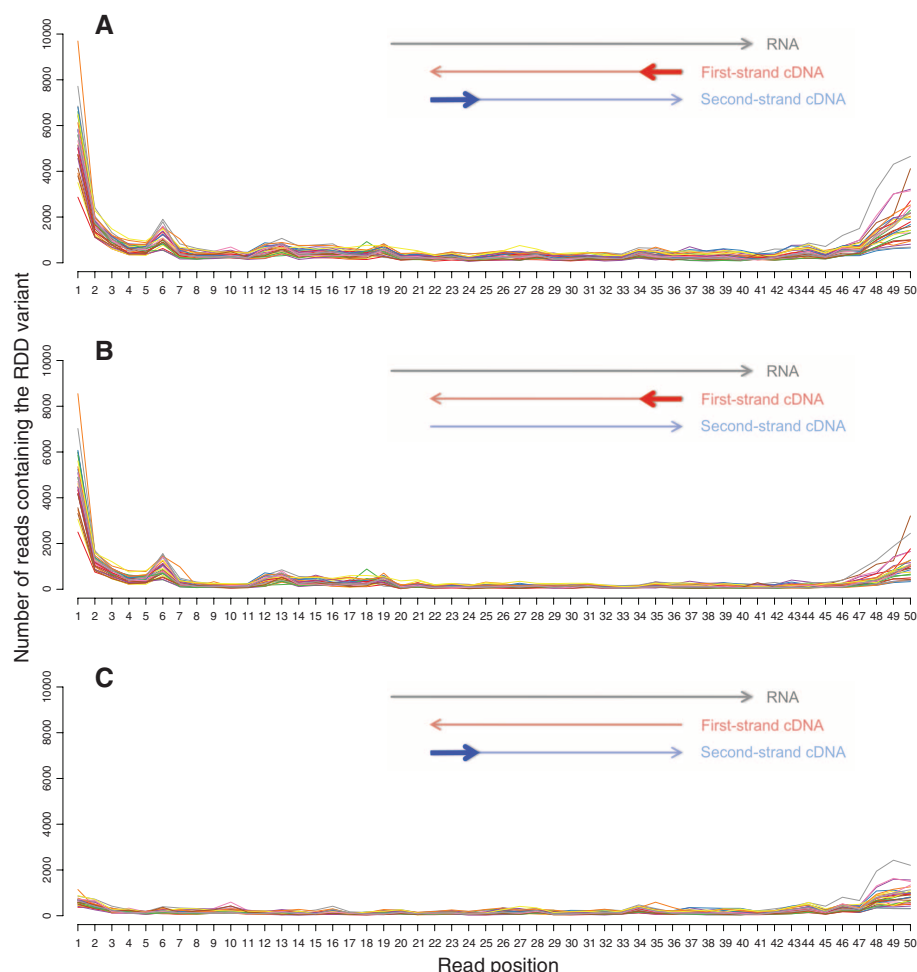


Fig. 2. Distribution of RDD variants across read positions. **(A)** We took all reads used in (1) that carry the variant type of RDDs and examined the position of RDD variants in the reads. Each of the 27 individuals is shown in a different color. The same analysis was repeated separately for **(B)** reads derived from the 5' end of the first-strand cDNA (opposite to the RNA strand) and **(C)** reads derived from the 5' end of the second-strand cDNA (same as the RNA strand). In the inset diagrams, red and blue thick arrows highlight the orientation of reads used in analysis.

reads and recalled the RDDs. As a result, a vast majority (8364, 82%) of the 10,210 RDDs were eliminated.

In addition to read mapping-related issues, many RDD sites identified in (1) may actually be

heterozygous single-nucleotide polymorphisms (SNPs) that were incorrectly called as homozygous due to the low coverage of genome sequences (as few as four reads were used to determine homozygosity in the DNA). In fact, we identified 556

RDDs that are annotated in the most recent SNP database (dbSNP132), and 476 (~86%) of them match the SNP type. Because the SNP database is far from being comprehensive, it is likely that many other RDDs are also derived from heterozygous SNPs that are not called due to low genome sequence coverage.

Taken together, we identified 9037 (~89%) of 10,210 RDD sites that may be false positives derived from gene duplications, read-end misalignment, and/or SNPs. Although it is possible that some of the remaining 1173 sites are real RDDs, only 14.8% of them are A-to-G type, which is believed to be the most common type of editing. This low fraction probably indicates a high false-positive rate even in the remaining set of sites. We manually examined many of these positions, and a large fraction of the variant calls appeared to be derived from misalignments, particularly at the splicing junctions. In the future, it will be of paramount importance to confirm their presence with alternative methods to rule out additional artifacts. High-throughput sequencing will continue to revolutionize the field of RNA editing by delivering enormous amounts of data at single-base resolution (6, 7). However, such data have their intrinsic limitations and pitfalls (8) and should therefore be used with caution. Rigorous sequence data analyses are crucial to reliably identify RDDs that should then be subject to further experimental validations.

References and Notes

1. M. Li *et al.*, *Science* **333**, 53 (2011).
2. K. Nishikura, *Annu. Rev. Biochem.* **79**, 321 (2010).
3. V. Blanc, N. O. Davidson, *Wiley Interdiscip. Rev. Syst. Biol. Med.* **2**, 594 (2010).
4. W. J. Kent, *Genome Res.* **12**, 656 (2002).
5. B. Langmead, C. Trapnell, M. Pop, S. L. Salzberg, *Genome Biol.* **10**, R25 (2009).
6. M. L. Metzker, *Nat. Rev. Genet.* **11**, 31 (2010).
7. J. B. Li *et al.*, *Science* **324**, 1210 (2009).
8. F. Meacham *et al.*, *BMC Bioinformatics* **12**, 451 (2011).

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Response to Comments on “Widespread RNA and DNA Sequence Differences in the Human Transcriptome”

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Kleinman and Majewski, Pickrell *et al.*, and Lin *et al.* suggest that mapping and sequencing errors and genetic variants led to false discovery of RNA-DNA sequence differences in our paper. We repeated our analysis using two different sequence alignment methods and carried out additional experiments including whole genome DNA sequencing. The results are consistent with our finding of widespread RNA-DNA sequence differences.

We reported uncovering RNA sequences that differ from the underlying DNA sequences (1). With the RNA sequences and corresponding DNA sequences of B cells from 27 individuals, we found more than 10,000 sites where the RNA and DNA sequences differ. We termed these RNA-DNA differences (RDDs). Because DNA serves as the template for RNA

synthesis, systematic differences between DNA and RNA sequences are thought to be rare. The known mechanisms that can lead to such differences are RNA editings mediated by two families of deaminases, ADARs (adenosine deaminases that act on RNA) and APOBECs (apolipoprotein B mRNA editing enzymes, catalytic polypeptide-like), which lead to A-to-G and C-to-U changes. We found all 12 types of differences, including transversions that are not likely to be caused by known editing mechanisms. In addition to B cells, we found RDDs in other cell types,

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Table 1. Studies that identify RNA-DNA differences.

A. Studies that identify widespread RNA-DNA differences published after our paper (1)

Authors	System	Type (including A>G)	Technology
Bahn <i>et al.</i> , 2012 (4)	Human glioblastoma, breast cancer cell lines	All 12 types	Illumina RNA-Seq of whole transcriptome
Ju <i>et al.</i> , 2011 (3)	B cells of 17 Korean individuals	All 12 types	Illumina RNA-Seq of whole transcriptome
Silberberg <i>et al.</i> , 2012 (2)	Brain samples of patients and controls	9 types	454 high-throughput sequencing of mRNA of 7 genes
Wu <i>et al.</i> , 2011 (5)	Small RNAs from <i>Caenorhabditis elegans</i>	All 12 types	Illumina sequencing of small RNAs

B. Previous studies that demonstrate RNA-DNA differences beyond the known editing mechanisms

Authors	System	Type (including A>G)	Technology
Sharma <i>et al.</i> , 1994 (6)	Human and rat tissues	T>C	Sanger sequencing
Novo <i>et al.</i> , 1995 (17)	Multiple human tissues	T>A	Sanger sequencing
van Leeuwen <i>et al.</i> , 1998 (7)	Brain tissues from Alzheimer's and Down syndrome patients	ΔGA; ΔGT; ΔCT	Sanger sequencing
Bourara <i>et al.</i> , 2000 (18)	HIV-1 infected human T cells	G>A; C>U	Sanger sequencing
Qian <i>et al.</i> , 2002 (19)	Fibroblasts from patients	5 types	Sanger sequencing
Klimek-Tomczak <i>et al.</i> , 2006 (20)	Colorectal adenocarcinoma tissue in patients and controls	G>A	Sanger sequencing
Brulliard <i>et al.</i> , 2007 (21)	Cancer and normal human tissues	All 12 types	Computational analysis of expressed sequence tags; experimental validation by denaturing high-performance liquid chromatography
Martinez <i>et al.</i> , 2008 (22)	Human prostate cancer cell lines	A>G, G>A, C>T, T>C	Sanger sequencing
Ebhardt <i>et al.</i> , 2009 (23)	<i>Oryza sativa</i> and <i>Arabidopsis thaliana</i>	All 12 types	454 and Illumina sequencing of small RNAs
Grohmann <i>et al.</i> , 2010 (24)	Brain samples from psychiatric patients and controls	4 types	Sanger sequencing

including primary skin cells and brain tissues. By mass spectrometry, we showed that the RNA forms of RDDs are translated into proteins.

Since our publication, several groups (2–5) have published about finding RNA-DNA differences beyond the A-to-G and C-to-U types. In these projects, different sequencing platforms, tissue types, analysis methods, and inclusion criteria were used (Table 1A), thus supporting the conclusion that RDDs are more common than previously known. We also found examples in the literature where mutations (like RDDs) were found in the RNA and not the DNA of the patients; these have been implicated in human diseases, including Wilms' tumor (6) and Alzheimer's disease (7) (see Table 1B for additional examples). These findings of systematic differences between RNA and their corresponding DNA sequences beyond the known RNA editing events show that there are unknown steps involved in how DNA is copied into RNA. It also reveals additional genetic variation that extends beyond DNA sequence polymorphisms.

Kleinman and Majewski (8), Pickrell *et al.* (9), and Lin *et al.* (10) raise concerns about our analysis and suggest that mapping and sequencing errors, as well as genetic variation, led to false positives in our study. Their Comments do not refute the presence of RDDs; rather, the disagreement is over how many there are. We view the discovery of RDDs as the important point and find the exact number to be less salient, because it depends on thresholds and sample sizes. Just as in single-nucleotide polymorphism (SNP) discovery, a comprehensive list requires a large sample; we do not intend for our initial report to be a complete catalog of RDDs; rather, our goal is to show that the phenomenon exists. Here, we repeated our analysis using two different sequence alignment algorithms (GSNAP and BLAT), explained the basis for the characteristic features of RDD sites in sequence reads, and carried out additional sequencing (with next-generation and Sanger sequencings) as well as protein immunoblot analysis to support our conclusions.

First, we analyzed the sequence data on the 27 individuals using a different alignment program, GSNAP (11). Among the 10,210 RDD sites, 8069 (79%) were also identified by GSNAP. The correlation of RDD levels obtained by Bowtie and GSNAP was very high ($r = 0.9$).

Second, we examined some of the unexpected features of the RDD sites in sequence reads. Authors of the Comments raised the concern that more than the expected number of RDD sites are found at ends of reads (positional bias) and reads in one direction (directional bias). We, too, were puzzled by this; but, upon close examination, we found that both features result from clustering of RDD sites, insertions/deletions (indels) that accompany the RDDs, and unannotated exon-exon junctions.

Previous studies showed that A-to-G sites tend to cluster (12–14); our results show that RDDs also cluster. Among the identified RDD

sites, 2613 sites (26%) are within 25 nucleotides (nt), and 1059 sites (10%) are adjacent to each other (1). This is likely an underestimate, because only reads with two or fewer mismatches (in the seed) were included in our analysis (Fig. 1A). Sites that are in strings of multiple RDDs would not have met our inclusion criteria. A-to-G editing events are known to be subject to hyperediting (13, 14); thus, they allow us to test our hypothesis that clustering of sites contributes to positional bias. We examined our results and indeed found that sites that show positional bias are enriched for A-to-G editing events. Forty-one percent of the sites found only in the first or last base of RNA sequencing (RNA-Seq) reads are A-to-G editing sites, whereas among all the RNA-DNA sequence differences, only 23% are A-to-G sites.

In addition, the indels that accompany RDDs and unannotated exon junctions contribute to positional and directional biases (Fig. 1, A and B). The reads that contain RDDs and insertion/deletions or unannotated exon junctions were removed because they exceeded the number of mismatches we allowed (Fig. 1). Only reads that happen to capture one or few RDDs in a string, or those with only the RDD sites near the ends of sequence reads but without the insertion/deletion, were kept (Fig. 1). To quantify the impact of indels on positional bias, we examined the GSNAP data, because GSNAP allows indels to be incorporated (the version of Bowtie we used did not allow indels). GSNAP indeed “rescued” reads

that were previously discarded due to indels, and in these “rescued” reads, the RDD sites are no longer at the ends of the reads (Fig. 2). The GSNAP results show that 1586 of the RDD sites are accompanied by at least one insertion/deletion within 5 bp of the RDD sites. Among them, 14% were found only in the first or last nucleotides of mapped reads, thus illustrating that indels contribute to the positional bias. The positions of RDDs in the sequence reads are explained by the clustering of RDD sites and the association of RDDs with indels and are not due to technical errors.

The authors of the Comments also questioned whether RDDs could be artifacts of paralogous genes. We ruled out this possibility in three ways: (i) by examining the examples listed by Pickrell and colleagues, (ii) by read-depth analysis of RDD sites relative to the rest of the genome, and (iii) by realigning the RDD sites and surrounding sequences back to the entire genome using BLAT (15).

First, we examined the cases that Pickrell and others used as examples of paralogous genes. They pointed out correctly that some of the genes, such as *AP2A2* in table 3 of our original publication, belong to a family of genes that share similar sequences. However, to lead to a false RDD call, the sequences of the other genes (paralogs) must correspond to the RNA sequences of the RDD sites; this is not the case for our results. The RNA form of the peptide (aspartic acid, D, coded by GAC) for *AP2A2* (table 3 of

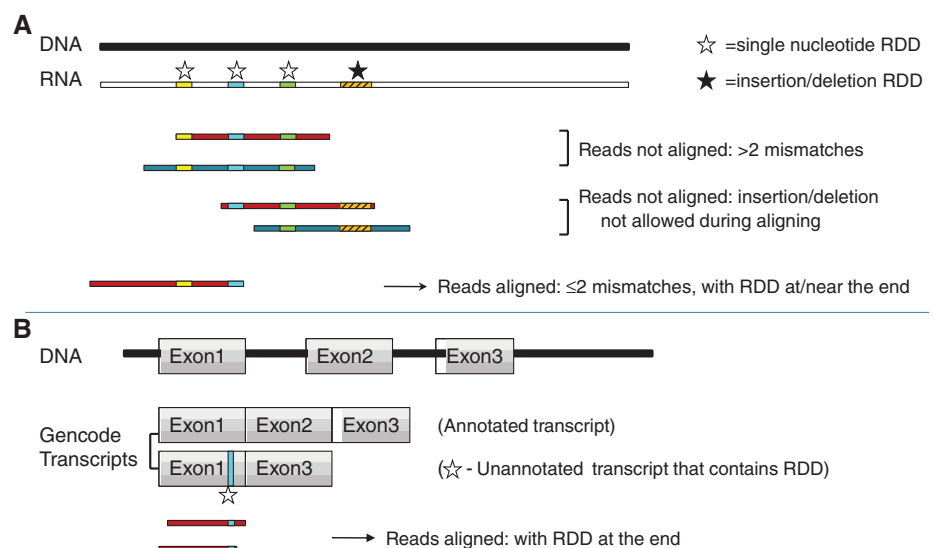
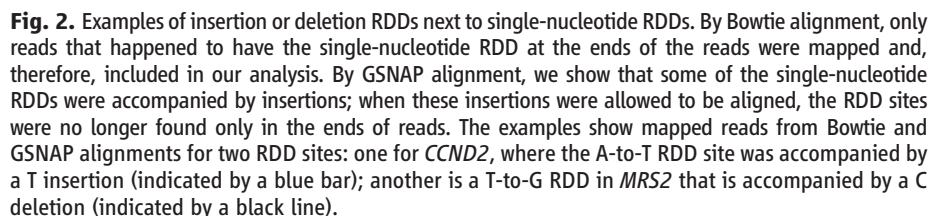
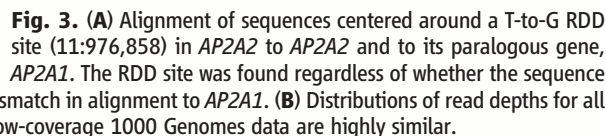


Fig. 1. Clustering of single-nucleotide RDDs, association of RDD sites with insertion/deletion RDDs, and unannotated exon-exon junctions led to positional and directional biases of RDDs in RNA-Seq reads. (A) In our Bowtie alignment, we allowed up to two mismatches (in the seed); thus, reads with multiple RDDs and/or insertion/deletion would not be aligned. Only those reads that happen to capture one or few of the RDDs that are clustered can be mapped; this only occurs when the RDDs are at the ends of the reads. (B) If reads represent exon-exon junctions that were not annotated by Gencode, they also would not be mapped by Bowtie. If a RDD site is found near such an unannotated junction, it can only be included if it is at the end of a read that does not contain the junction. The relative order of a single-nucleotide RDD with an insertion/deletion or an unannotated junction is important because reads that include the insertion/deletion or junction cannot be mapped; only reads in the direction that “meets” the single-nucleotide RDD have a chance to be included in the analysis.



To further examine whether the RDD sites arise due to misalignment to repetitive regions or paralogs, we took the 10,210 RDD sites and surrounding sequences (in three windows of 25, 50 and 75 nt 5' and 3' of each site) and aligned those sequences by BLAT to the genome. In this analysis, we examined sequences from the entire genome and not just the sequences corresponding to the Gencode genes; thus, if the original



analysis—which focused only on Gencode regions—led to misalignments, we would uncover them here. Among the 10,210 sites, 9289 mapped only

to the sites that we identified in Li *et al.* (1) (see Table 2). We examined the remaining 921 sites that mapped to additional genomic regions. We

found that 237 of them contain our RDD alleles, although in most of these cases, the match to the original sites had fewer mismatches than to the other locations; nevertheless, these could be mis-called as RDDs due to misalignment. Authors of the Comments suggest that any RDD that maps closely to another region in the genome is a false-positive finding. This is not correct; only sites that contain the identical sequences as our RDD alleles can lead to incorrect RDD calls. Based on BLAT results, less than 3% of the RDD sites may be miscalled due to paralogous sequences. This relatively low percentage is not surprising; in the original analysis, to avoid miscalling RDDs due to misalignment, we removed all sites in repetitive regions of the genome.

Table 2. BLAT analysis of RDD-containing sequences.

Query size (nt)	No. of matched* sequences (in addition to original location)	No. of sites that contain RDD allele† (in addition to original location)
51 (RDD site + 25 nt window)	921	237‡
101 (RDD site + 50 nt window)	538	138‡
151 (RDD site + 75 nt window)	286	75‡

*See supporting online material. †In most of these cases, the RDD sequences are better matched (with fewer mismatches) to the original locations than to the “other” locations. ‡There are overlaps among these three groups; of them, there are 330 unique RDDs that can be explained by DNA sequences.

Table 3. Additional sites sequenced by Sanger sequencing. (Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.)

Gene	Location (RDD type)			
ACIN1	chr14:22606709 (C-to-G)	chr14:22606941 (C-to-G)	chr14:22607009 (G-to-T)	chr14:22618623 (T-to-A)
AIM1	chr6:107124403 (A-to-T)			
AP2A2	chr11:976858 (T-to-G)			
AZIN1	chr8:103910812 (A-to-G)	chr8:103910813 (A-to-G)		
B2M	chr15:42791000 (G-to-C)	chr15:42791002 (C-to-G)	chr15:42791019 (G-to-C)	chr15:42791020 (A-to-T)
BLCAP	chr20:35580977 (A-to-G)			
C1orf63	chr1:25441492 (G-to-A)	chr1:25441498 (C-to-A)		
CLASP1	chr2:121813765 (G-to-A)			
CNN2	chr19:988983 (C-to-T)			
CPNE1	chr20:33678725 (A-to-C)	chr20:33678726 (T-to-C)		
DAP	chr5:10733501 (A-to-C)			
DCP1A	chr3:53297343 (A-to-C)			
EIF4G2	chr11:10775281 (T-to-C)			
ENO3	chr17:4800624 (T-to-G)			
FABP3	chr1:31618424 (T-to-A)			
FTHL16	chr11:61488612 (G-to-A)	chr11:61488613 (A-to-G)		
HNRNPU	chr1:243085469 (A-to-G)	chr1:243085472 (T-to-G)		
HSPA4	chr5:132459768 (T-to-G)			
KLRAQ1	chr2:48595587 (T-to-C)			
LSP1	chr11:1870029 (A-to-C)			
MAPK8IP1	chr11:45880558 (G-to-A)			
NACA	chr12:55392932 (G-to-A)	chr12:55392945 (T-to-C)	chr12:55392946 (G-to-A)	
NDUFA13	chr19:19500007 (G-to-A)	chr19:19500008 (C-to-A)		
NDUFC2	chr11:77468303 (C-to-G)			
PLEKHO1	chr1:148397845 (G-to-A)			
PNPLA6	chr19:7513741 (C-to-T)			
POLR1E	chr9:37476086 (T-to-C)			
POP1	chr8:99237725 (T-to-G)			
RPL23	chr17:34259865 (T-to-A)	chr17:34259866 (T-to-A)	chr17:34259867 (A-to-G)	chr17:34259877 (A-to-C)
RPL28	chr19:60590466 (G-to-A)	chr19:60590467 (T-to-A)		
RPS24	chr10:79469965 (C-to-A)			
RPS27	chr1:152230681 (C-to-T)	chr1:152230683 (A-to-T)	chr1:152230727 (C-to-G)	chr1:152230731 (A-to-C)
	chr1:152230743 (C-to-A)	chr1:152230743 (C-to-A)	chr1:152230746 (C-to-A)	
RPS6	chr9:19366252 (T-to-A)	chr9:19366257 (T-to-C)		
SCD5	chr4:83770861 (C-to-T)	chr4:83770862 (C-to-A)		
SEC16A	chr9:138455418 (A-to-G)	chr9:138455444 (A-to-G)		
SFPQ	chr1:35422397 (A-to-T)	chr1:35422398 (T-to-C)		
TCEB2	chr16:2761989 (A-to-C)			
TNFRSF14	chr1:2481920 (G-to-C)			
TUBB2C	chr9:139257264 (T-to-C)	chr9:139257297 (G-to-A)		
TUFM	chr16:28761607 (C-to-A)	chr16:28761608 (G-to-A)	chr16:28761609 (A-to-G)	
UQCRCQ	chr5:132231273 (T-to-C)	chr5:132231279 (C-to-A)		
TOR1AIP1	chr1:178144365 (T-to-C)			

The authors of the Comments were also concerned about unknown SNPs. They proposed that some of the RDDs can be explained by non-reference DNA sequences at the sites. The samples used are those studied extensively by several consortia, including HapMap and 1000 Genomes, that focus on identifying DNA sequence variants; thus, unknown SNPs are unlikely. The Comment authors pointed out correctly that some of the RDD sites are known SNPs. As noted in our original publication, we used known SNP sites only if the genotypes of our samples are homozygous based on results from the HapMap and the 1000 Genomes projects. These studies used different technologies to obtain the genotypes, so we are confident of the results. To address this concern experimentally, we validated the DNA sequences corresponding to several hundred RDD events by Sanger sequencing and deep sequenced the DNA of two individuals by next-generation sequencing.

In addition to the Sanger sequencing we carried out previously (1), here we sequenced an

additional 73 sites in two to five individuals (average of 3) (Table 3 and Fig. 4). These sites were picked mostly randomly, except for some genes that are of particular interests to us, including the site in RPL28. Over the few hundred sites that were sequenced, two of the sites (chr7:73173273 and chr1:199732016) differ in sequences from 1000 Genomes/ Reference genome data. The remaining ones have identical sequences as those in 1000 Genomes/ Reference genome. Thus the discordance rate is less than 1%.

In addition, we carried out whole-genome sequencing of the DNA of two subjects, GM12004 to 60X and GM12750 to 30X coverage. We asked whether deep- and whole-genome sequencing would allow us to identify DNA variants that led to misidentification of RDD alleles. We checked the DNA sequences of these two individuals at their RDD sites: 48 (10%) of the 486 RDD events in GM12004 and 84 (6%) of 1350 RDD events in GM12750 have one or more DNA reads that contain the RDD alleles (Table 4). In most cases (>60%), less than 5% of DNA reads

contain the RDD alleles. Even if we eliminated all events with one or more DNA reads that correspond to the RDD alleles, however, we would only decrease the number of RDDs by ~10%. This confirms that DNA sequence errors or unknown DNA sequence variants do not lead to considerably more false-positive RDDs.

Last, we carried out immunoblotting to validate our mass spectrometry results. We showed that a T-to-A RDD site in *RPL28* leads to a loss of a stop codon and a 55-amino acid extension of the RPL28 protein. In our original publication (1), we showed mass spectra for the extended peptides that result from that RDD. Subsequently, we raised an antibody against the extended peptide and confirmed that the RNA form of RPL28 is expressed at protein level with an expected size of 13.7 kD (Fig. 5).

In summary, we reported the unexpected finding that there are many sites where RNA sequences differ from the underlying DNA sequences. In response to the Comments, we showed that clustering of RDDs and insertions/deletions that accompany RDDs explain why RDD and A-to-G editing sites are found more often at the ends of sequencing reads than expected. We also carried out read-depth analysis, realignment with BLAT, and examination of individual examples to show that paralogs and copy number variations do not contribute considerably to false RDD identifications. We also provided additional Sanger and next-generation sequencing data, as well as immunoblot results, to validate the RDD sites. With results from these analyses and recent reports from other groups (2–4), we are certain that there are many more mismatches between RNA and their underlying DNA sequences than previously known. Next, it will be important to uncover the mechanisms that lead to RDDs and study their functional impact.

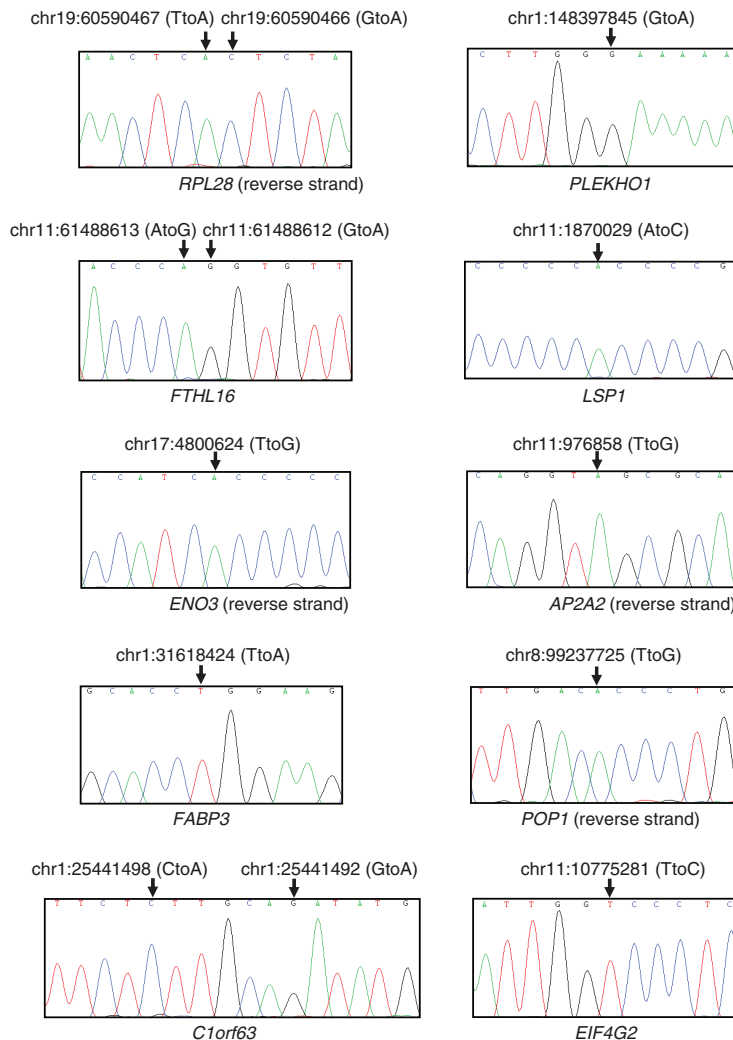


Fig. 4. Examples of Sanger sequencing traces of genomic DNA sequences at RDD sites in Table 3. The results confirm DNA sequences used in our analysis.

References

1. M. Li *et al.*, *Science* **333**, 53 (2011).
2. G. Silberberg, D. Lundin, R. Navon, M. Öhman, *Hum. Mol. Genet.* **21**, 311 (2012).
3. Y. S. Ju *et al.*, *Nat. Genet.* **43**, 745 (2011).
4. J. H. Bahn *et al.*, *Genome Res.* **22**, 142 (2012).
5. D. Wu, A. T. Lamm, A. Z. Fire, *Nat. Struct. Mol. Biol.* **18**, 1094 (2011).
6. P. M. Sharma, M. Bowman, S. L. Madden, F. J. Rauscher 3rd, S. Sukumar, *Genes Dev.* **8**, 720 (1994).
7. F. W. van Leeuwen *et al.*, *Science* **279**, 242 (1998).
8. C. L. Kleinman, J. Majewski, *Science* **335**, 1302 (2012). www.sciencemag.org/cgi/content/full/335/6074/1302-c
9. J. K. Pickrell, Y. Gilad, J. K. Pritchard, *Science* **335**, 1302 (2012). www.sciencemag.org/cgi/content/full/335/6074/1302-d
10. W. Lin, R. Piskol, M. H. Tan, J. B. Li, *Science* **335**, 1302 (2012). www.sciencemag.org/cgi/content/full/335/6074/1302-e
11. T. D. Wu, S. Nacu, *Bioinformatics* **26**, 873 (2010).
12. S. Osenberg, D. Dominissini, G. Rechavi, E. Eisenberg, *RNA* **15**, 1632 (2009).
13. S. Carmi, I. Borukhov, E. Y. Levanon, *PLoS Genet.* **7**, e1002317 (2011).
14. A. Athanasiadis, A. Rich, S. Maas, *PLoS Biol.* **2**, e391 (2004).
15. W. J. Kent, *Genome Res.* **12**, 656 (2002).
16. C. Alkan *et al.*, *Nat. Genet.* **41**, 1061 (2009).

Table 4. Examples of DNA and RNA sequences at RDD sites

A. Examples of RDD sites by type											
Site	Gene	Type	Id	DNA-Seq				RNA-Seq*			
				#A	#C	#G	#T	#A	#C	#G	#T
chr12:264669	KDM5A	A-to-C	GM12004	115	0	0	0	10	<u>3</u>	0	0
chr12:107563438	CORO1C	A-to-G	GM12004	100	0	0	0	12	<u>0</u>	<u>3</u>	0
chr1:26674340	HMG2	A-to-T	GM12750	21	0	0	0	24	0	0	<u>5</u>
chr10:79469965	RPS24	C-to-A	GM12750	0	30	0	0	<u>18</u>	2	0	0
chr9:130493973	SET	C-to-G	GM12750	0	39	0	0	<u>0</u>	25	<u>4</u>	0
chr15:70835705	ADPGK	C-to-T	GM12750	0	49	0	0	0	40	0	<u>6</u>
chr9:35046559	VCP	G-to-A	GM12004	0	0	108	0	<u>5</u>	0	14	0
chr11:57262732	TMX2	G-to-C	GM12750	0	0	42	0	0	<u>5</u>	36	0
chr1:21168554	EIF4G3	G-to-T	GM12750	0	0	35	0	0	0	15	<u>2</u>
chr7:44807753	PPIA	T-to-A	GM12004	0	0	0	80	<u>3</u>	0	0	17
chr12:131882519	GOLGA3	T-to-C	GM12004	0	0	0	114	0	<u>2</u>	0	10
chr2:175646325	ATF2	T-to-G	GM12750	0	0	0	55	0	0	<u>2</u>	14

B. Potential RDD sites miscalled due to incorrect DNA sequences by type											
Site	Gene	Type†	Id	DNA-Seq				RNA-Seq*			
				#A	#C	#G	#T	#A	#C	#G	#T
chr2:37181538	EIF2AK2	A-to-G	GM12004	87	0	1	0	4	0	<u>10</u>	0
chr3:172263105	TNIK	A-to-T	GM12004	73	1	0	1	9	0	0	<u>1</u>
chr14:22313163	SLC7A7	C-to-A	GM12750	1	59	0	0	<u>2</u>	16	0	0
chr1:31680044	SERINC2	C-to-G	GM12750	0	19	19	0	0	7	<u>5</u>	0
chr11:118394263	RPS25P8	C-to-T	GM12750	0	47	0	2	0	28	0	<u>8</u>
chr21:33853781	SON	G-to-A	GM12750	24	0	0	0	<u>47</u>	0	0	0
chr9:113735965	UGCG	G-to-C	GM12004	0	20	27	0	0	<u>2</u>	10	0
chr19:40898882	ZBTB32	G-to-T	GM12750	0	0	0	58	0	0	0	<u>111</u>
chr5:133560909	PPP2CA	T-to-A	GM12004	1	0	0	60	<u>2</u>	0	0	12
chr1:74943861	CRYZ	T-to-C	GM12750	0	1	0	19	0	<u>2</u>	0	14
chr6:7234323	SSR1	T-to-G	GM12750	0	0	1	38	0	0	<u>2</u>	12

*RDD allele in bold and underlined. †Some of the types, such as A-to-C, did not show any possible miscalls; therefore, they are not included in Table 3B.

17. F. J. Novo, A. Kruszewski, K. D. MacDermot, G. Goldspink, D. C. Górecki, *Nucleic Acids Res.* **23**, 2636 (1995).

18. K. Bourara, S. Litvak, A. Araya, *Science* **289**, 1564 (2000).

19. L. Qian, E. V. Quadros, A. Regec, J. Zittoun, S. P. Rothenberg, *Blood Cells Mol. Dis.* **28**, 134; discussion, 143 (2002).

20. K. Klimek-Tomczak *et al.*, *Br. J. Cancer* **94**, 586 (2006).

21. M. Brulliard *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 7522 (2007).

22. H. D. Martinez *et al.*, *J. Biol. Chem.* **283**, 29938 (2008).

23. H. A. Ebhardt *et al.*, *Nucleic Acids Res.* **37**, 2461 (2009).

24. M. Grohmann *et al.*, *PLoS ONE* **5**, e8956 (2010).

25. H. Li *et al.*; 1000 Genome Project Data Processing Subgroup, *Bioinformatics* **25**, 2078 (2009).

Supporting Online Material

www.sciencemag.org/cgi/content/full/335/6074/1302-f/DC1

Methods

References

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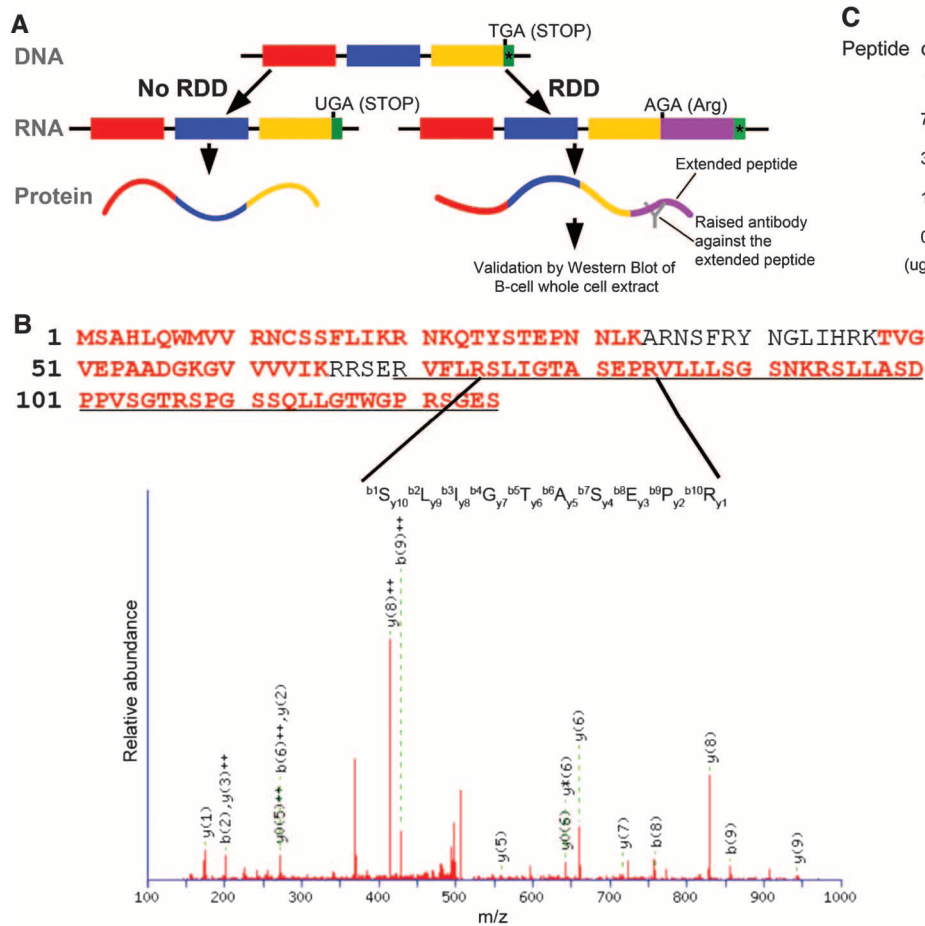


Fig. 5. Validation of RPL28 RDD (chr19:60590467, T>A) using an antibody specific for the RNA form of RPL28. **(A)** Schematic diagram of the validation procedure. **(B)** Detection of the RNA form of RPL28 by mass spectrometry, as reported in (1). The sequence colored in red shows the peptides detected by liquid chromatography–tandem mass spectrometry, and the underlined sequence corresponds to the extended 55 amino acids that resulted from the T>A RDD and subsequent loss of a stop codon. The representative mass spectra are shown. **(C)** Dot blot confirms that the RPL28 antibody detects the RPL28 peptide (extended peptide in the RNA form) specifically, but not the negative control peptide. **(D)** Western blot shows that the specific antibody detects RPL28 protein from human B cells. (Lane 1) Input lysate for the mass spectrometry analysis (pooled sample). (Lane 2) Lysate from one individual.

ORNITHOLOGY

Capping a Survey of All Extant Birds

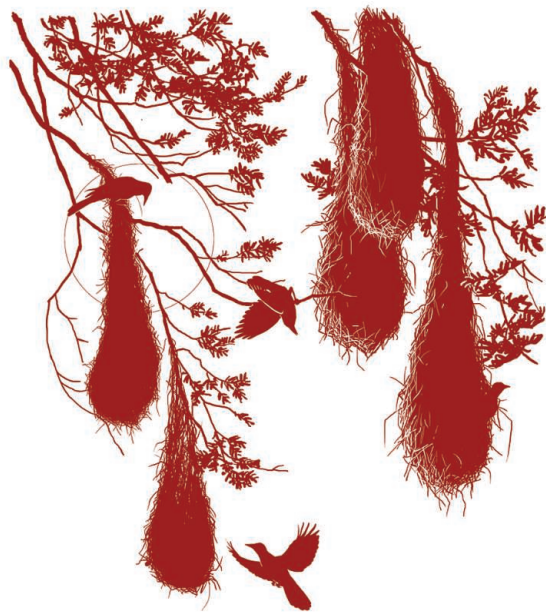
Kimball L. Garrett

Illustrated handbooks and family accounts of birds have appeared regularly, but none has had so ambitious a goal as the *Handbook of the Birds of the World* (HBW). The publication of its 16th volume, a milestone in the ornithological literature, completes a series begun in 1992 that treats each of the world's circa 10,000 bird species with a detailed account and color illustration, provides a specialist-authored summary chapter on the systematics and comparative biology of each family, and employs abundant color photography. The cumulative heft of this endeavor is impressive: 12,564 pages, 1035 color plates, and 6386 color photos—weighing some 67 kg.

The family accounts treat systematics, morphology, habitat, habits, vocalizations, food and feeding, breeding, movements, relationship with humans, and conservation status. Individual species accounts include similar categories, along with a range map and bibliography. For the fourth and subsequent volumes, an introductory essay treats an aspect of ornithology, with subjects weighted toward classification, taxonomy and nomenclature, and conservation but also addressing migration, bioacoustics, and other topics. This final volume opens with an essay by Anders Pape Møller on climate change and birds.

In a midcourse change in 2002, the handbook was expanded to 16 volumes (from an originally intended 12), and more details were included in subsequent species accounts. Oddly, vocalizations were not covered at all in the first three volumes—thus omitting voice descriptions for the wonderfully vocal tinamous, galliforms, and procellariiforms—and were treated only for selected families (e.g., cuckoos, owls, and nightjars) in the next three. The inclusion of voice descriptions for all species began with the last nonpasserine volume.

Planning HBW was a monumental undertaking, with some 175 authors having to be



Substantial investments. Oropendolas (*Psarocolius*) spend weeks weaving pendent elongate bags and then months of nesting and postfledging care.

shepherded. A key drawback of the 20-year production was the need to “lock in” to a taxonomic authority early on. Intensified molecular work since the series began has led to important shifts in family and even ordinal composition, rendering the HBW family-level taxonomy particularly obsolete in this last volume, as noted below.

Volume 16 treats four closely related families within the massive radiation of “New World nine-primaried oscines”: the tanagers (Thraupidae), New World sparrows (Emberizidae), cardinal-grosbeaks (Cardinalidae), and blackbirds and orioles (Icteridae). (The other main component of this radiation—the wood-warblers, Parulidae—was covered in the preceding volume.) The volume surveys 762 species (in 182 genera), with all but 48 species (7 genera) found only in the New World. These include some of the most colorful of all birds, arguably culminating in the tanager genus *Tangara*—a stunning collision of explosive speciation and a preposterous palette of pigment and pattern. They also include “Darwin’s

finches” (the famous Galápagos radiation of, it turns out, tanagers, not finches); such superabundant blackbirds as the widespread and well-studied red-winged blackbird (*Agelaius phoeniceus*) and the great-tailed grackles (*Quiscalus mexicanus*) that noisily gather in every zócolo of Middle America; the iconic northern cardinal (*Cardinalis cardinalis*); and the white-crowned sparrow (*Zonotrichia leucophrys*), subject of innumerable studies on vocal dialects, migration, reproductive physiology, and environmental adaptation. The tanagers display a wide range of morphological variation and foraging behaviors—especially when a host of genera treated here under Emberizidae are transferred to Thraupidae. The remaining emberizids and the cardinalids are relatively more uniform, but the final family, the Icteridae, is remarkably diverse in its social systems, nesting habits, and foraging behavior. These blackbirds, orioles, and relatives occupy most New World habitats, from forests of all types to grasslands, marshes, and desert scrub.

Taxonomic issues arise frequently in the volume. Besides the impending wholesale transfer of many “emberizid” genera into the Thraupidae, there are the removals from Thraupidae of *Piranga* and relatives (to Cardinalidae) and *Euphonia* (to Fringillidae). That the volume does not reflect these changes adds an awkward tone to some of the family-level discussions of comparative biology. The species-level taxonomy was left to the whim of family account authors; that of the emberizid chapter differs in many respects from taxonomy of the American Ornithologists’ Union even though the author is a member of AOU’s committee on classification and nomenclature. The treatment of Savannah sparrow (*Passerculus sandwichensis*) seems especially odd: two nearly indistinguishable saltmarsh taxa on the California

coast are not only assigned to different species but one even is subsumed into a single, widespread continental subspecies. The split of the fox sparrow (*Passerella iliaca*) into four species is a matter of taxonomic “taste.” Regardless of one’s preference, it is unfortunate that here the call notes of two component species—“Slate-colored” (*P. schistacea*) and “Thick-billed” (*P. megarhyncha*)—are stated to be identical. In fact, the highly distinct calls are among the best means of separating the two taxa in the field. Such quibbles are minor because the accounts discuss differing taxo-

Handbook of the Birds of the World

Volume 16, Tanagers to New World Blackbirds

Josep del Hoyo, Andrew Elliott, and David A. Christie, Eds.

Lynx Edicions, Barcelona, 2011.
893 pp. €212, \$280, £178.
ISBN 9788496553781.

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onomic treatments at great length. I have nothing but praise for all the authors for their thorough and readable considerations of a morass of biological information.

Having led the way with birds, Lynx Edicions is currently producing a mammal handbook (two of a projected eight volumes have been completed). But one must turn to the Internet to find as comprehensive a treatment for fish (1) or amphibians (2, 3).

The publisher has not abandoned the *HBW* vein. An additional volume in preparation will provide a comprehensive index to the

series along with accounts of the more than 50 species described since the publication of their respective volumes. Also planned is a synoptic, two-volume global checklist—with illustrations and range maps for all species, in an updated taxonomy. Lastly, and inevitably, *HBW* content will be available and updated online for an annual subscription fee.

An invaluable reference, *Handbook of the Birds of the World* opens a majestic window into the ornithological literature. Ultimately, however, its greatest contribution may be as a fountain of raw material about the diver-

sity and biogeography of birds for present and future biologists as well as amateur ornithologists. Through its illustrations, displays of geographic ranges, and outlines of basic life history information, it is bound to stimulate all who work with birds in the field, the museum, or the armchair.

References and Notes

1. www.fishbase.org.
2. <http://amphibiaweb.org>.
3. <http://research.amnh.org/vz/herpetology/amphibia>.

10.1126/science.1219019

COMPUTER SCIENCE

Stunningly Successful Solutions

Paul Curzon

Computer science is a young subject that has been around a very long time. Its origins go back millennia, but it has only started to take on a life of its own since the 1940s. John MacCormick's *Nine Algorithms That Changed the Future* offers a great way to find out what computer science is really about. In this very readable book, MacCormick (a computer scientist at Dickinson College) shows how a collection of sets of intangible instructions invented since the 1940s has led to monumental changes in all our lives.

Despite the name, computer science is the study of computation, not just computers. Most people barely wonder how computers do the things they do or even notice how amazing achieving those things often is. How can it be that in a fraction of a second a search engine searches billions of Web pages and comes back with the right one? Even today, computers aren't that fast. Few people have any idea about the often stunningly elegant computation that lies behind such accomplishments. That computation boils down to an algorithm: a step-by-step procedure that, if followed precisely, guarantees some desired result is achieved. That result could be the answer to a calculation, the address of a Web page that was searched for, or confirmation that money has been transferred between bank accounts. Algorithms solve problems for people.

Computer scientists have a very distinctive idea of what it means to solve a problem. You

might claim to have solved a puzzle (such as a crossword or a Rubik's Cube) if you manage to finish it. However, they would agree only when you have a step-by-step procedure that would produce the correct solution when applied blindly to any member of the same class of puzzle. In their view, problems are solved when we have algorithms that tackle them. It is only then that a computer can take over and deliver answers for us.

The derivation of the word algorithm suggests the actual age of the discipline of computer science. The term dates back to a Muslim scholar from the 9th century, al-Khwārizmī. The word comes from the title of one of his books, an account of algorithms invented by Indian mathematicians that are even older still. Mathematicians have been developing algorithms for pretty much as long as they have been doing math. For example, Euclid and Newton give their names to algorithms they invented that are still used today. Ada Lovelace is widely credited with writing (in 1843) the first algorithm in a form suitable for a machine to execute: a program. It is only with the advent of working computers in the 1940s that the study of algorithms, and so computer science, really took off. That's because computers allow algorithms to do useful things for humans without those humans needing to do the work. What has followed from the humble beginnings of Lovelace's first program is truly amazing, and MacCormick describes some of the most remarkable of those computer science inventions.

Despite its title, the book covers many

more than just nine algorithms. It actually explores nine problem areas where the algorithms derived by computer scientists have entered everyday use. These programming solutions are constantly serving all of us—in our mobile phones, in our banking transactions, when we download music, and more. They do so without most people even realizing that they are there. Their efficiency and effectiveness have led to them just being taken for granted. Some, such as

those involved in digital signatures, achieve things that appear at first sight to be plainly impossible: for example, turning strings of 1s and 0s that anyone can copy at will into a digital signature that cannot possibly be forged. MacCormick discusses other algorithms that pull off their tricks with amazing speed and accuracy, such as the collection working behind the scenes of search engines—instantly pulling needles out of the haystack that is the Internet, day in day out. Yet others give computers their amazing ability to get things right. The algorithms called “error correcting codes,” for example, allow computers to transmit extensive streams of data without getting a single number wrong even when the data are constantly being distorted.

These stunningly effective algorithms allow computers to achieve their seeming miracles: results that extend far beyond the abilities of any human even though the instructions themselves are the work of humans. MacCormick provides a taste of why we computer scientists get so excited about algorithms—for their utility, of course, but also for their beauty and elegance. On reading *Nine Algorithms That Changed the Future*, you too may become infected with the computer science bug.

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Nine Algorithms That Changed the Future
The Ingenious Ideas That Drive Today's Computers
by John MacCormick
Princeton University Press,
Princeton, NJ, 2012.
231 pp. \$27.95, £19.95.
ISBN 9780691147147.

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Navigating the Anthropocene: Improving Earth System Governance

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Science assessments indicate that human activities are moving several of Earth's sub-systems outside the range of natural variability typical for the previous 500,000 years (1, 2). Human societies must now change course and steer away from critical tipping points in the Earth system that might lead to rapid and irreversible change (3). This requires fundamental reorientation and restructuring of national and international institutions toward more effective Earth system governance and planetary stewardship.

We propose building blocks of such a new institutional framework, based on a comprehensive assessment conducted in 2011 by the Earth System Governance Project, a 10-year social science-based research program under the auspices of the International Human Dimensions Programme on Global Environ-

mental Change (IHDP) (4, 5). The assessment has been designed to contribute to the 2012 United Nations (UN) Conference on Sustainable Development in Rio de Janeiro, which will focus on the institutional framework for sustainable development and possible reforms of the intergovernmental governance system.

The assessment revealed remaining differences of opinion among social scientists, as well as an increasing consensus in many areas. As a general conclusion, our work indicated that incremental change (6)—the main approach since the 1972 Stockholm Conference on the Human Environment—is no longer sufficient to bring about societal change at the level and with the speed needed to mitigate and adapt to Earth system transformation. Structural change in global governance is needed, both inside and outside the UN system and involving both public and private actors.

To this end, decision-makers must seize the opportunity in Rio to develop a clear and ambitious roadmap for institutional change and effective sustainability governance within the next decade. Seven reform measures are urgently required as a first step.

Seven Building Blocks

First, the environmental agencies and programs of the United Nations must be reformed and/or upgraded (7). Many reform proposals have been submitted in recent decades. Some of the more radical proposals—such as an international agency that centralizes and integrates existing intergovernmental organizations and regimes—are unlikely to be implemented and would yield uncertain gains. However, most of us see substantial benefits in upgrading the UN Environment Programme to a UN specialized agency for environmental protection along the lines of the World Health Organization or the International Labor Organization, that is, a strong environmental organization with a sizable role in agenda-setting, norm-development, compliance manage-

The United Nations conference in Rio de Janeiro in June is an important opportunity to improve the institutional framework for sustainable development.

ment, science assessment, and capacity-building (8–10).

Second, it is crucial to strengthen the integration of the social, economic, and environmental pillars of sustainable development, from local to global levels. The UN Commission on Sustainable Development (CSD) was created in 1992 for this purpose. Yet its political relevance as a subbody to the UN Economic and Social Council has remained limited. Governments must now take action to improve the integration of sustainable development policies. In our view, the CSD must be replaced by a new mechanism that stands much higher in the international institutional hierarchy. The most promising route is creating a high-level UN Sustainable Development Council directly under the UN General Assembly (11). To be more effective, such a council should rely not on traditional UN modes of geographical representation, but give special predominance to the largest economies—the Group of 20—as primary members that hold at least 50% of the votes in the council. Only such a strong novel role for the Group of 20 will allow the UN Sustainable Development Council to have a meaningful influence in areas such as economic and trade governance. The countries that cooperate in the Group of 20 represent about two-thirds of the world's population and around 90% of global gross national product. This legitimizes a sizeable institutional role for these nations as primary members of a powerful UN Sustainable Development Council.

Third, better integration of sustainability governance requires governments to close remaining regulatory gaps at the global level. One such area is the development and deployment of emerging technologies, such as nanotechnology, synthetic biology, and geo-engineering. Such emerging technologies promise significant benefits, but also pose major risks for sustainable development. They need an international institutional arrangement—such as one or several multilateral framework conventions—to support forecasting, transparency, and information-sharing; further develop technical standards; help clarify the applicability of existing treaties; promote public discussion and input; engage multiple stakeholders in policy dia-

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logues, and ensure that environmental considerations are fully respected (12).

Fourth, integration of sustainability policies requires that governments place a stronger emphasis on planetary concerns in economic governance. Environmental goals must be mainstreamed into global trade, investment, and finance regimes so that the activities of global economic institutions do not undermine environmental treaties because of poor policy coherence (13, 14). Changes in world trade law to discriminate between products on the basis of production processes are critical if investments in cleaner products and services are to be encouraged, for example, through special recognition for environmentally friendly products and technologies. Such discrimination, however, must be based on multilateral agreement to prevent protectionist impacts.

Fifth, we argue for a stronger reliance on qualified majority voting to speed up international norm-setting. Political science research shows that governance systems that rely on majority-based rule are quicker to arrive at far-reaching decisions and that consensus-based systems limit decisions to the preferences of the least ambitious country (15). Yet at the international level, majority-based decision-making is still rare and needs to be further extended especially when Earth-system concerns are at stake. Weighted voting mechanisms can ensure that decisions take all major interests among governments into account without granting veto power to any country (16).

Sixth, stronger intergovernmental institutions as outlined here raise important questions of legitimacy and accountability (17). Global governance through UN-type institutions tends to give a larger role to international and domestic bureaucracies, at the cost of national parliaments and the direct involvement of citizens. Accountability can be strengthened when stakeholders gain better access to information and decision-making through special rights enshrined in

agreements or stronger participation in councils that govern resources and in commissions that hear complaints. Greater transparency can help empower citizens and consumers to hold governments and private actors accountable and can provide incentives for better sustainability performance (18). In particular, stronger consultative rights for civil society representatives in intergovernmental institutions would be a major step forward, including in the UN Sustainable Development Council that we propose. This requires, however, transparent and effective accountability mechanisms for civil society representatives vis-à-vis their constituencies, as well as appropriate mechanisms that account for imbalances in the strength of civil society among different countries and for power differentials among different segments of civil society (for example, through separate sub-chambers for different regions and/or different interests, such as environmentalists, industry, youth, and so on).

Seventh, equity and fairness must be at the heart of a durable international framework for sustainable development. Strong financial support of poorer countries remains essential (19). More substantial financial resources could be made available through novel financial mechanisms, such as global emissions markets or air transportation levies for sustainability purposes (20).

Constitutional Moment

The world saw a major transformative shift in governance after 1945 that led to the establishment of the UN and numerous other international organizations, along with far-reaching new international legal norms on human rights and economic cooperation. We need similar changes today, a “constitutional moment” in world politics and global governance.

Such a reform of the intergovernmental system—which is at the center of the 2012 Rio Conference—will not be the only level of societal change nor the only type of action

that is needed toward sustainability. Changes in the behavior of citizens, new engagement of civil society organizations, and reorientation of the private sector toward a green economy, are all crucial to achieve progress. Yet, in order for local and national action to be effective, the global institutional framework must be supportive and well designed. We propose a first set of much-needed reforms for effective Earth system governance and planetary stewardship. The 2012 Rio Conference offers an opportunity and a crucial test of whether political will exists to bring about these urgently needed changes.

References and Notes

1. W. Steffen *et al.*, *Global Change and the Earth System* (Springer, New York, 2004).
2. H. J. Schellnhuber *et al.*, Eds., *Earth System Analysis for Sustainability* (MIT Press, Cambridge, MA, 2004).
3. J. Rockström *et al.*, *Nature* **461**, 472 (2009).
4. F. Biermann, *Glob. Environ. Change* **17**, 326 (2007).
5. Science and Implementation Plan of the Earth System Governance Project (Working papers, Earth System Governance, 2009); www.earthsystemgovernance.org.
6. Incremental change refers to minor reforms that attempt to increase efficiency and effectiveness of governance without fundamentally altering decision-making rules, basic organizational arrangements, funding levels, or legal commitments, among others.
7. O. R. Young, L. A. King, H. Schroeder, Eds., *Institutions and Environmental Change* (MIT Press, Cambridge, MA, 2008).
8. F. Biermann, *Environment* **42**, 22 (2000).
9. S. Charnovitz, *Columbia J. Environ. Law* **27**, 323 (2002).
10. A. Najam, M. Papa, N. Taiyab, *Global Environmental Governance* (International Institute for Sustainable Development, Winnipeg, Canada, 2007).
11. Earth System Governance Project, Eds., *Towards a Charter Moment: Hakone Vision on Governance for Sustainability in the 21st Century* (International Environmental Governance Architecture Research Group, Tokyo, 2011).
12. K. W. Abbott, G. E. Marchant, D. J. Sylvestre, *Environ. Law Rep.* **36**, 10931 (2006).
13. P. Newell, in *Re-thinking Development in a Carbon-Constrained World*, E. Palosuo, Ed. (Ministry for News Anal. Foreign Affairs, Finland, Laivastokatu, 2009), pp. 184–195.
14. J. Gupta, N. van der Grijp, Eds., *Mainstreaming Climate Change in Development Cooperation* (Cambridge Univ. Press, Cambridge, UK, 2010).
15. J. Hovi, D. F. Sprinz, *Glob. Environ. Polit.* **6**, 28 (2006).
16. Weighted voting implies a departure from the traditional intergovernmental approach of “one-country-one-vote,” which gives equal weight to all countries regardless of, e.g., their population size. Our proposal of special voting rights for the Group of 20 in the UN Sustainable Development Council is one example. Another example is the double-weighted majority voting in the treaties on stratospheric ozone depletion, which accept majority decisions in certain areas as long as they include the majority of all developing countries and the majority of industrialized countries.
17. S. Bernstein, *J. Int. Law Int. Rel.* **1**, 139 (2005).
18. A. Gupta, Ed., *Glob. Environ. Polit.* **10**, 1 (2010).
19. World Bank, *World Development Report 2010: Development and Climate Change* (World Bank, Washington, DC, 2009).
20. B. Müller, *International Adaptation Finance* (Oxford Institute for Energy Studies, Oxford, 2008).

10.1126/science.1217255

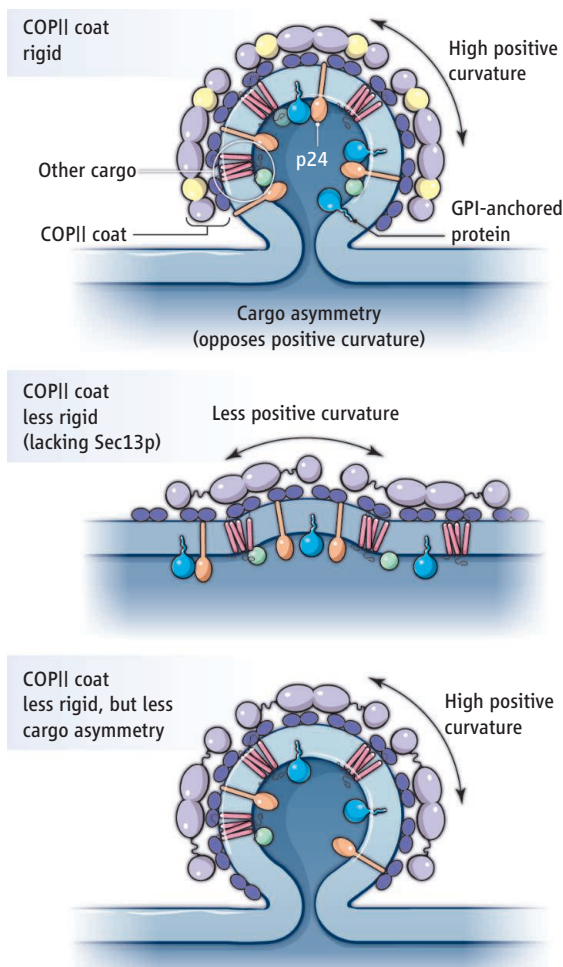
Membrane Bending Tug of War

John Silvius

The surfaces of biological membranes are very crowded places (1, 2). “Steric pressure” created by interactions between membrane proteins can influence important aspects of membrane behavior, including the lateral diffusion of membrane proteins (3), the architecture and function of cell adhesion proteins (4), and cargo selection in clathrin-mediated endocytosis (5, 6). On page 1359 of this issue, Čopić *et al.* (7) propose that surface-crowding effects can also influence the bending of membranes into highly curved structures such as transport vesicles.

In eukaryotic cells, membrane and soluble proteins synthesized in the endoplasmic reticulum (ER) and destined for export to the Golgi are recruited to specialized “ER exit sites” where they are packaged into transport vesicles surrounded by a cage-like protein assembly known as a COPII coat (8). To form the outermost layer of the COPII coat, the proteins Sec13p and Sec31p assemble into well-defined polyhedral structures (9). This rigid architecture is thought to constrain the assembly of other coat components that directly interact with the membrane, thereby promoting the membrane bending required to form a transport vesicle (see the figure).

Sec13p, although not essential for COPII coat assembly, forms extensive contacts with Sec31p that are predicted to enhance the rigidity of the Sec13p-Sec31p scaffold and the membrane-deforming ability of the coat (9). Accordingly, although Sec13p is essential for viability in budding yeast, Čopić *et al.* found that Sec13p-deficient yeast regained viability if they expressed a mutated form of Sec31p designed to restore rigidity to COPII coats lacking Sec13p. Sec13p-deficient yeast also regain viability upon deletion of any of a small group of so-called “bypass-of-sec-thirteen” (*BST*) genes (10). To determine how *BST* gene deletions restore viability to cells lacking Sec13p, Čopić *et al.* extensively screened the yeast



Opposing forces. (Top) To form membrane transport vesicles at ER exit sites, yeast cells require a rigid COPII coat (violet/yellow) to bend the membrane outward against the asymmetric pressure (favoring inward curvature) created by locally concentrating luminal-asymmetric cargo like GPI-anchored and p24-family proteins. **(Middle)** COPII coats without Sec13p lack sufficient rigidity to bend the membrane against this resistance. **(Bottom)** The membrane-bending ability of COPII coats lacking Sec13p is restored by depleting luminal-asymmetric cargo, thereby reducing membrane bending resistance, at exit sites.

(*Saccharomyces cerevisiae*) genome for *BST* genes. Most genes they identified (some known previously) fall into two groups. One group encodes four of the eight known yeast members of the p24 protein family, transmembrane proteins that cycle between the ER and Golgi and are required for export of glycosylphosphatidylinositol (GPI)-anchored proteins from the ER (8). The other

Membrane protein crowding may influence the biological processes that drive vesicle formation.

group comprises four genes encoding enzymes that remodel protein-linked GPI residues, introducing modifications that are essential for concentrating GPI-anchored proteins at ER exit sites (11).

Čopić *et al.* noted that both GPI-anchored proteins and p24-family proteins are highly abundant in the ER membrane and are highly asymmetric, presenting most or (for GPI-anchored proteins) all of their mass at the luminal face of the ER membrane. Proteins of both families normally concentrate at ER exit sites, where their local abundance and asymmetric structures create excess steric pressure on the luminal side of the ER membrane that resists the outward membrane bending required to form a transport vesicle. The authors propose that COPII coats without Sec13p, unlike normal coats, lack sufficient rigidity to drive membrane bending against this opposing resistance. Depleting luminal-asymmetric membrane proteins from ER exit sites should reduce this resistance, potentially enough to allow even Sec13p-deficient COPII coats to generate transport vesicles. Čopić *et al.* show that this can indeed be achieved either by reducing expression of p24-family proteins (12) or by blocking the concentration of GPI-anchored proteins at ER exit sites [which additionally blocks the accumulation of p24-family proteins at these sites (13)].

The model of Čopić *et al.* offers an intriguing possible explanation for the mechanistic origins of the *BST* (and *SEC13*) gene-deletion phenotypes. Given that deletion of almost any of the identified *BST* genes reduces the concentration of p24-family proteins at ER exit sites, it would be intellectually satisfying (but may be technically challenging) to demonstrate that membrane budding at these sites can be modulated by modifying asymmetric steric pressure without perturbing local accumulation of p24-family proteins. In this regard, it is encouraging that the authors report that an additional *BST* gene encodes Env29p, a transmembrane protein enriched at ER exit sites that binds

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luminal cargo proteins to form a luminal-asymmetric complex; this species does not interact with p24-family proteins.

The model of Čopič *et al.* raises important questions about the potential biological importance of steric-crowding effects (particularly for highly asymmetric membrane proteins) in other processes that entail membrane deformation. To what degree might steric interactions of asymmetric membrane proteins influence the energetics, and even the mechanism, of membrane bending at other steps in vesicle trafficking or other membrane functions? Can local concentration of asymmetric membrane proteins promote, rather than hinder, membrane bending in some contexts, such as in the bud-

ding of enveloped viruses from the plasma membrane? Steric pressure in biological membranes should also cause highly asymmetric proteins to spontaneously distribute unequally between membrane regions with different local curvatures. Such effects could play a physiological role in sorting of asymmetric membrane proteins during trafficking. There are clearly many places to look for new and important ways in which the crowded nature of membranes may influence their biological functions.

References

1. S. Takamori *et al.*, *Cell* **127**, 831 (2006).
2. A. D. Dupuy, D. M. Engelman, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 2848 (2008).
3. M. J. Saxton, K. Jacobson, *Annu. Rev. Biophys. Biomol.*

Struct. **26**, 373 (1997).

4. K. D. Patel, M. U. Nollert, R. P. McEver, *J. Cell Biol.* **131**, 1893 (1995).
5. M. S. Bretscher, J. N. Thomson, B. M. Pearce, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4156 (1980).
6. P. Bhagatji, R. Leventis, J. Comeau, M. Refaie, J. R. Silvius, *J. Cell Biol.* **186**, 615 (2009).
7. A. Čopič *et al.*, *Science* **335**, 1359 (2012).
8. J. Dancourt, C. Barlowe, *Annu. Rev. Biochem.* **79**, 777 (2010).
9. S. Fath, J. D. Mancias, X. Bi, J. Goldberg, *Cell* **129**, 1325 (2007).
10. M. J. Elrod-Erickson, C. A. Kaiser, *Mol. Biol. Cell* **7**, 1043 (1996).
11. G. A. Castillon, R. Watanabe, M. Taylor, T. M. Schwabe, H. Riezman, *Traffic* **10**, 186 (2009).
12. M. Marzioch *et al.*, *Mol. Biol. Cell* **10**, 1923 (1999).
13. G. A. Castillon *et al.*, *Mol. Biol. Cell* **22**, 2924 (2011).

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PHYSIOLOGY

She Said No, Pass Me a Beer

Troy Zars

Sex is widely recognized as rewarding across the animal kingdom, but rejection of sexual advances or even deprivation can have negative effects. Behavioral studies indicate that sex can be positive—that is, viewed as a reward—in some learning experiments. Rejection of a sexual advance can also have lasting effects on physiology and behavior. How the success or failure of courtship behaviors are linked to other behaviors has been difficult to address. On page 1351 of this issue Shohat-Ophir *et al.* (1) assess the connection between the rewarding properties of sex and the effects of sex deprivation in the fly *Drosophila melanogaster*. The authors discover a neural system defined by a specific neuropeptide that unexpectedly couples courtship rejection or sex deprivation to a rewarding behavior—ethanol consumption.

What happens when a male fly courts a

female? In most laboratory experiments, two flies are put into a cramped empty space with bright lights and a camera focused on every move (2, 3). Perhaps surprisingly under these conditions, the male will almost immediately pursue the female, using all of his courtship tools to entice her to copulate. A male will vibrate one wing in a love song, tap her abdomen with his foreleg, and touch her genitalia with his proboscis (a structure that includes some olfactory and taste sense organs), and if the conditions are right, attempts at copulation will not be rejected. The courting takes less than 10 min under optimal conditions; copulation lasts about 20 min. Afterward, the two flies part ways, she to lay her eggs and he to court again.

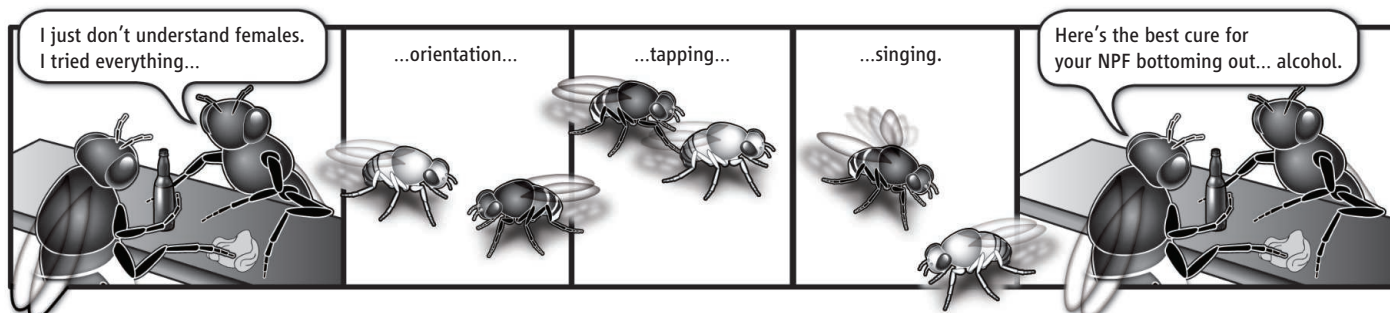
Shohat-Ophir *et al.* investigated whether the rejection of courtship advances would alter a different rewarding behavior—ethanol consumption. Reward seeking is a fundamental behavior in which an animal will do something to make things better, such as eating when hungry. When given a choice

Sexual rejection or deprivation is connected to ethanol consumption in *Drosophila*.

between food with 15% ethanol and normal food slurry, mated male flies consumed about equal amounts of either type (“mated” males were those combined with virgin females at a ratio of 1:5 for several hours, with a new cohort of females every day for 4 days). By contrast, when male flies were rejected (non-virgin female flies strenuously reject new copulation attempts), the males later showed a strong preference for the ethanol-spiked food. This preference was also evident in virgin male flies and males that had been in the presence of decapitated virgin females (males will court a headless female, but copulation attempts are rarely successful) (3). If, however, rejected males copulated at a later

Neural system for reward behavior. After an elaborate courtship ritual by a male *Drosophila*, rejection by a female, or sexual deprivation, reduces the amount of neuropeptide F in the male fly brain. This correlates with the male’s increased preference for ethanol, a behavior associated with reward and reward seeking.

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time, the ethanol preference disappeared. Thus, rejection or deprivation of sex leaves male flies in a state that increases the preferential consumption of ethanol. Copulation overrides the deprived state and ethanol preference is reduced.

In mammals (e.g., rats and mice), neuropeptide Y (NPY) influences ethanol-related behaviors (4). For example, elimination of NPY expression in the mouse increases ethanol consumption. The homolog of NPY in *Drosophila* is neuropeptide F (NPF) (5, 6). In no animal model has the NPF/NPY neural system connected sexual experience to ethanol-related behaviors. Shohat-Ophir *et al.* discovered that courtship rejection reduced the amount of NPF produced in the male fly brain. They also found that reduced signaling by the NPF receptor (by decreasing NPF receptor expression with RNA interference) resulted in males that preferred ethanol-spiked food even after mating (these flies did not have the NPF signaling effect of sex). Extrinsic activation of NPF-expressing neurons (by triggering the opening of TRPA1 cation channels) in virgin

males decreased their preference for ethanol intake—ethanol intake was similar to that of males that had previously copulated.

Could the NPF neural circuit in the fly brain be part of a reward system? Pairing of ethanol exposure (at inebriating concentrations) with an odor leads to a long-term memory and preference for that odor in *Drosophila*, suggesting that ethanol experience is rewarding (7). Shohat-Ophir *et al.* observed that pairing of male flies with virgin female flies in the presence of an odor (there is presumably some copulation taking place) led to a later preference of those males for the odor. These results suggest that sex is rewarding. Extrinsic activation of the NPF-expressing neurons in the presence of an odor, the same technique that decreases ethanol consumption, increases flies' preference for that odor. The data of Shohat-Ophir *et al.* suggest that the NPF neural circuit is part of a reward system that adjusts reward-seeking behavior (ethanol intake) appropriately.

Although it is titillating to think about the relationship between spurned advances and

ethanol consumption (anthropomorphizing the results from flies is difficult to suppress, but the relevance to human behavior is obviously not yet established), the study of Shohat-Ophir *et al.* study should not be taken lightly. The authors provide new insights into a neural circuit that links a rewarding social interaction with a lasting change in behavior preferences. Identifying the NPF system as critical in this linkage offers exciting prospects for determining the molecular and genetic mechanisms of reward and could potentially influence our understanding of the mechanisms of drugs of abuse.

References

1. G. Shohat-Ophir, K. R. Kaun, R. Azanchi, U. Heberlein, *Science* **335**, 1351 (2012).
2. L. C. Griffith, A. Ejima, *Learn. Mem.* **16**, 743 (2009).
3. J. C. Hall, *Science* **264**, 1702 (1994).
4. T. E. Thiele, D. J. Marsh, L. Ste. Marie, I. L. Bernstein, R. D. Palmiter, *Nature* **396**, 366 (1998).
5. T. Wen, C. A. Parrish, D. Xu, Q. Wu, P. Shen, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 2141 (2005).
6. R. S. Hewes, P. H. Taghert, *Genome Res.* **11**, 1126 (2001).
7. K. R. Kaun, R. Azanchi, Z. Maung, J. Hirsh, U. Heberlein, *Nat. Neurosci.* **14**, 612 (2011).

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GEOPHYSICS

Monitoring Volcanoes

R. S. J. Sparks,¹ J. Biggs,¹ J. W. Neuberg²

The ascent of magma in volcanoes is typically accompanied by numerous small earthquakes, the release of magmatic gases, and surface deformation (1). Systematic volcano monitoring to detect these phenomena began in 1845 with the completion of the Osservatorio Vesuviano. Other volcano observatories soon followed, such as the Hawaiian Volcano Observatory, which celebrates its 100th anniversary this year. Today, the World Organization of Volcano Observatories has 80 members. The range and sophistication of the detection systems has increased dramatically, and advanced models of volcanic processes are helping to interpret monitoring data. Yet, key problems remain both with distinguishing volcanoes that will erupt from those that will not and with global data coverage.

Seismic signals remain a key aspect of volcano monitoring. As magma moves toward the Earth's surface, stress changes in the volcanic edifice, as well as magma rup-

ture and stick-slip motion of the magma body, lead to highly regular seismic patterns, often referred to as volcanic tremor (2). The signals are typically very weak and may be missed by regional networks, requiring a dedicated network of seismometers near the volcanic edifice. Seismic monitoring is therefore at the heart of every volcano observatory.

Early attempts to interpret seismic signals on volcanoes used methods adopted from earthquake seismology. Simple event counts or amplitude estimates were used as crude indicators for the level of volcanic activity. In the past 20 years, broadband seismic sensors have enabled detection of seismic signals from volcanic earthquakes in a wide frequency range, allowing volcano seismologists to distinguish between different types of volcanic events and to attribute different signals to different volcanic processes (3). Conceptual models help to detect and quantify magma or fluid movements, or to identify stress changes in the volcanic edifice. Hence, short-term forecasting can be achieved by interpreting systematic changes in seismic energy release as changes in magma ascent rates and changes in seismic patterns and

Despite technological advances, volcano monitoring around the world is woefully incomplete.

spectral characteristics as indicators of critical changes in magma properties.

Compared with global seismology, where data exchange is routine, volcano observatories are more independent and less willing to share data. Particularly during a crisis, raw seismic data are often confidential, such that only the local observatory can give advice. Some observatories have established links to research institutions. However, it is crucial that advice to authorities is channeled through the observatories or official scientific advisory committees; maverick interpretations from outside groups can be a problem.

Most volcanic eruptions are preceded and accompanied by ground deformation. Methods to measure surface movements include high-precision leveling, electronic distance measurement with lasers, ground tiltmeters, and—in the past 20 years—the Global Positioning System (4). These methods are typically used in combination. Strain meters in boreholes (5), one of the world's most sensitive geophysical instruments, are used at very few volcanoes. Deformation data were long interpreted with a simple point source model, the Mogi model (6), but today's numeri-

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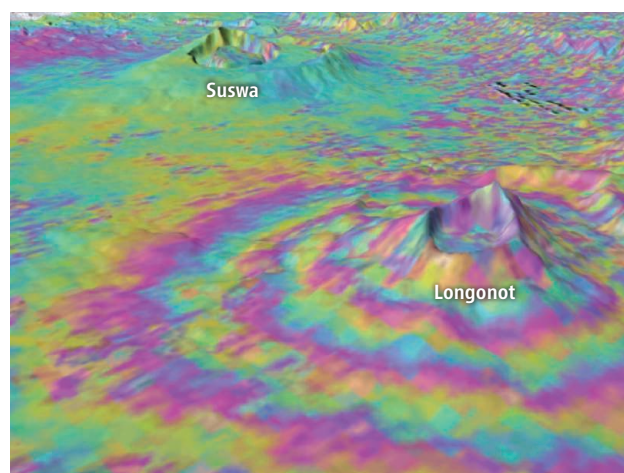
cal models incorporate representations of crustal rheology and of differently shaped pressure sources (7). With these new models, the shape and depth of the magma chamber can be determined more precisely.

Satellite-based methods provide a route to detecting unrest on currently unmonitored volcanoes (8). Interferometric synthetic aperture radar (InSAR) uses the phase component of radar images to determine the position of the Earth's surface. Simultaneously recorded images from different radars produce digital elevation models, vital for predicting the path and run-out of pyroclastic flows and lahars, and time-separated images measure deformation. The radar beam can pass through clouds. This is particularly useful in the tropics, where cloud cover frequently obscures visual observations.

The satellite view provides a global perspective, mapping tectonic strain across continents and allowing the exploration of volcanoes in remote, underfunded, or inaccessible environments. As a result, the list of volcanoes previously thought to be dormant but now known to be showing signs of unrest (see the figure) is growing rapidly. These observations enable targeting of resources for more detailed, ground-based monitoring.

Despite its enormous potential, InSAR is still a young endeavor. Technical issues, such as repeat times and mission longevity, are in the hands of the space agencies. In a promising development, the European Space Agency's (ESA) Sentinel satellites, due for launch in 2013, are expected to acquire data over all land masses every 6 days for the next 20 years. Attaching radar systems to unmanned aerial vehicles (UAVs) promises greater flexibility and bespoke acquisition plans, although flight paths are more complex than those of satellites, complicating the data interpretation. The challenges will be, first, to distinguish between magmatic, hydrothermal, and even atmospheric errors, and second, to determine which processes will culminate in eruption.

Measurements of volcanic gas composition and fluxes, as well as temperature, have long been stalwarts of monitoring. Until recently, these measurements were made on fumaroles and hot springs, sometimes placing scientists at high risk. In situ data remain very valuable, but there has been prodigious progress in remote measurements from ground-based instruments and satellites. Analytical



Not dormant. This InSAR image shows a pulse of uplift during 2004 to 2006 at Mount Longonot, Kenya, a volcano previously believed to be dormant. The image, from the ESA satellite Envisat, is draped over a digital elevation model from the Shuttle Radar Topography Mission. Each complete color cycle (fringe) represents 2.8 cm of displacement toward the satellite (14). The distance between craters is ~35 km.

petrological estimates, notably from melt inclusion studies, have advanced understanding of gas inventories (9). Networks of ultraviolet spectrometers and imaging cameras provide detailed sulfur dioxide (SO_2) time series that can be combined with seismic and deformation data to give a much more complete and informative picture of volcano behavior (10). Likewise, SO_2 and thermal data are now measured from satellites (11), although it is proving hard to get agreement between the ground-based and satellite measurements (12). Furthermore, SO_2 data are often difficult to interpret: A decrease in SO_2 may mean that the threat of eruption is diminishing because of decreased magma supply or that the gas is being trapped at depth and the threat is increasing.

Other novel monitoring techniques include infrasound and portable ground radar, which are already being deployed to document explosive eruptions and ash clouds. Muon tomography holds promise for imaging the interior of lavas, and UAVs may provide new monitoring capability, but both methods will need considerable development to become widely used.

Despite these technological advances and the increasing integration of different data types, early warning of eruptions still faces major challenges. The most important issue is how to tell whether a period of volcanic unrest will lead to eruption. There are more cases of unrest that do not lead to eruption than those that do. False alarms are one of the most problematic issues for observatories. Evacuations that are called but then nothing happens can undermine public trust, whereas evacuations

that are called too late or not at all can lead to tragedy. Volcanic systems are likely to fail suddenly. Sometimes, very minor differences in system properties determine whether failure and eruption occur or not. The exact timing of eruptions may be difficult to predict, and it is even likely that some volcanic systems are inherently unpredictable. Monitoring can provide insights into patterns and consequences of activity that can help draw evacuation plans.

Furthermore, most volcanoes around the world are not monitored effectively or at all. A study of 441 active volcanoes in 16 developing countries (13) reveals that 384 have rudimentary or no monitoring, including 65 volcanoes identified as posing a high risk to large populations. Satellite systems such as InSAR can provide regional, or even global, data but are rarely applied in real time. This unsatisfactory situation is to some extent ameliorated by rapid response teams at the request of countries during volcanic emergencies, notably the Volcanic Disaster Assistance Program of the U.S. Geological Survey. But even well-funded observatories suffer from a wide gap between research developments and their implementation as forecasting tools on an operational level. Developing capacity and closing these gaps is a priority for the volcanological community.

References and Notes

1. R. S. J. Sparks, *Earth Planet. Sci. Lett.* **210**, 1 (2003).
2. R. M. Iverson et al., *Nature* **444**, 439 (2006).
3. J. W. Neuberg, in *Encyclopedia of Solid Earth Geophysics*, H. K. Gupta, Ed. (Springer, Berlin/Heidelberg, 2011), vol. 1, pp. 261–269.
4. D. Dzulisin, *Rev. Geophys.* **41**, 1001 (2003).
5. A. T. Linde, K. Agustsson, I. S. Sacks, R. Stefansson, *Nature* **365**, 737 (1993).
6. K. Mogi, *Bull. Earthq. Res. Inst. Univ. Tokyo* **36**, 99 (1958).
7. S. Hautmann et al., *J. Geophys. Res.* **115**, (B9), B09203 (2010).
8. M. E. Pritchard, M. Simons, *Nature* **418**, 167 (2002).
9. M. Edmonds, *Phil. Trans. R. Soc. A* **366**, 4559 (2008).
10. P. A. Nadeau, J. L. Palma, G. P. Waite, *Geophys. Res. Lett.* **38**, L01304 (2011).
11. M. Rix et al., *Sel. Topics Appl. Earth Observations Remote Sens* **2**, 196 (2009).
12. B. T. McCormick, M. Edmonds, T. A. Mather, S. A. Carn, *Geochem. Geophys. Geosyst.*, 10.1029/2011GC003945 (2012).
13. W. Aspinall et al., *Bristol University Cabot Institute and NGI Norway for the World Bank: NGI Report 20100806*, 3 (2011).
14. J. Biggs, E. Y. Anthony, C. J. Ebinger, *Geology* **37**, 979 (2009).
15. We thank S. Carn for helpful comments on volcanic gas monitoring. J.B. is supported by the National Centre for Earth Observation and by an ESA Support to Science Element Fellowship.

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APPLIED PHYSICS

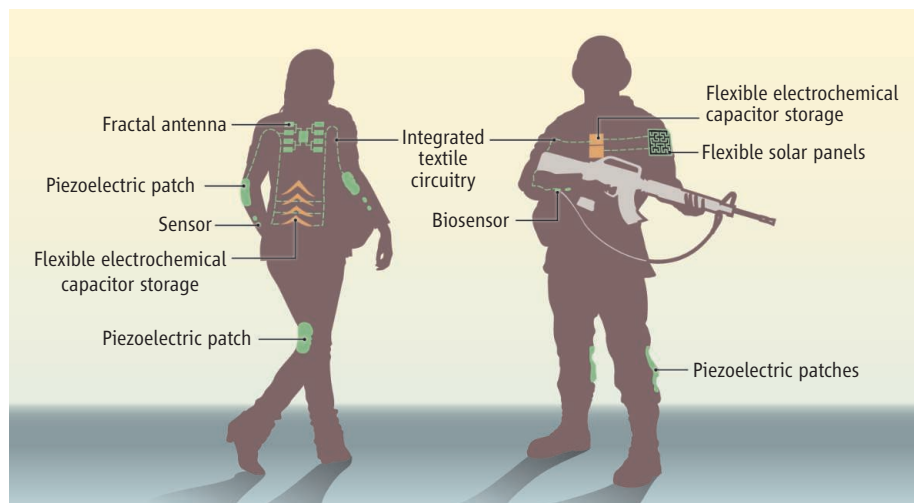
Valuing Reversible Energy Storage

A process based on laser-converted graphene is used to fabricate high-value energy storage material.

John R. Miller

The development of new materials that provide the capability of high-performance energy storage combined with flexibility of fabrication opens up the possibility of a wide range of technological applications. On page 1326 of this issue, El-Kady *et al.* (1) describe thin and highly flexible electrochemical capacitors (ECs) that were created by means of a very simple and innovative process. Unlike the usual approaches of making thin graphene electrodes that start with a particulate and use roll-coating, screen printing, or ink-jet printing (2), their process involves focusing a low-power laser onto a thin graphene oxide deposit to convert it into graphene. The incorporation of graphene in electrodes created with mechanical processes tends to be in agglomerates that provide little performance advantage over traditional particulate-activated carbon electrodes. El-Kady *et al.*'s approach also contrasts with plasma-assisted chemical vapor deposition processes that have been used to grow vertically oriented graphene nanosheet electrodes (3). Although graphene structures grown by such methods are well-formed and offer performance advantages over traditional activated carbon materials, they require complicated vacuum process equipment, plus the graphene growth rate is very slow (4). The somewhat simple EC electrode fabrication process reported by El-Kady *et al.* therefore appears to circumvent many of the difficulties encountered with traditional processes.

Whenever a new energy storage technology is reported, almost inevitably the first question asked and the first data cited focus on its "watt-hours per kilogram" (Wh/kg) value. This measure refers to specific energy, a metric that dates historically to the days when heavy batteries provided almost the only means available to store electrochemical energy. Despite, and almost in defiance of, the emergence of newer energy storage technologies, however, specific energy continues to be referenced without further consideration as the most important characteristic of any new energy storage technology, the gold standard of its worth or value (5). This



Power dressing. Design concept for self-powering "smart" garments, outfitted with piezoelectric patches to harvest energy from body movements and flexible electrochemical capacitors to store the energy. Power to camouflage uniforms is one possible use.

is all wrong—specific energy is only one of many metrics by which the value of storage technology can be measured. Indeed, it may be one of the least important when it comes to assessing its value for use in today's newest and most innovative applications (6).

Usually the second question asked is the cost, the most popular metric being "cost per unit of energy" (\$/kWh). The table lists the three types of capacitors and two different battery technologies, using dollars per kilowatt-hour as the cost metric (7). The value for electrostatic capacitors (metalized-film capacitors) is \$2.5 million per kWh. Electrolytic capacitors cost \$1 million per kWh. Curiously despite such extremely high costs, both technologies are found in almost every piece of electronics available today. Much lower in cost, at a mere \$20,000 per kWh, are ECs (8). But the stark comparison to lithium-ion batteries at \$1000 or lead acid bat-

teries at \$150 per kWh suggests that \$/kWh is not actually a very important metric in some decision-making. Cost is but one of the many ways by which storage technology can be measured, and again it may be one of the least important when it comes to assessing the value of an energy storage technology for use in applications.

Reversibility, essentially the efficiency of a round-trip cycle that first stores then later uses the stored energy, is also an important metric for energy storage technology in many present and emerging applications. Unlike money that may be deposited in an honest savings bank that later is totally returned and often with interest, energy deposited in any storage device has associated losses that prevents the full return. Then the question is what fraction of the deposited energy will eventually be returned. This strongly depends on the storage media as well as the rate at which energy is stored, the storage time, and the rate at which energy is extracted (9). Unlike batteries that typically have higher losses during charge than during discharge, ECs can be totally charged and discharged very quickly with high efficiency. Energy reversibility is often a most important factor in establishing the value of a storage technology for many of today's energy conservation applications.

Cycle life goes hand-in-hand in importance with energy reversibility. Some energy

DEVICE PRICE LIST	
Technology	Cost (\$/kWh)
Electrostatic capacitor	2,500,000
Electrolytic capacitor	1,000,000
Electrochemical capacitor ("super" or "ultracapacitor")	20,000
Li-ion battery	1000
Lead acid battery	150

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conservation applications, for example, regenerative energy capture during the stopping of a hybrid city transit bus, may require more than 1 million charge/discharge cycles during their operational life. A storage system can be replaced several times or “supersized” to reduce the depth of discharge in each cycle and increase cycle life, practices commonly used for battery technologies. However, both approaches mean that the storage system will have higher cost. ECs, by contrast, rely on physical rather than chemical storage and do not suffer from limitations of cycle life. They effectively can be “right-sized” at the start and last the entire life of a given application. In short, cycle life can impart great value to an energy storage technology.

Storage system shape is another factor that may have high value in some applications. Energy density advantages generally can be best achieved with shapes approaching a cube, whereas power density advantages can

be best achieved with thin, large-area designs. A given energy storage technology may lend itself to either one of these extremes. Besides offering power advantages, very thin energy storage devices may enable a broad range of new applications. If these devices have mechanical flexibility, then there are even more potential applications. One example is a fabric EC that is charged by piezoelectric transducers that harvest and store body-movement energy. This would allow the creation of “smart” garments for making fashion statements or to power flexible electronics that may be embedded in a military uniform (see the figure). Other examples include energy storage for use in camouflage, for car interiors, and to make electronic wall paper.

An interesting feature of the conversion process reported by El-Kady *et al.* is that graphene patterns can be “laser scribed” directly onto very thin graphene oxide deposits (10). As one example, interdig-

tated planar structures of graphene can be created, which after receiving an electrolyte overcoat become planar ECs. This route for producing extremely thin and highly flexible energy storage structures is quite exciting and shows great promise.

References and Notes

1. M. F. El-Kady, V. Strong, S. Dubin, R. B. Kaner, *Science* **335**, 1326 (2012).
2. L. T. Le, M. H. Ervin, H. Qiu, B. E. Fuchs, W. Y. Lee, *Electrochem. Commun.* **13**, 355 (2011).
3. X. Shao *et al.*, *J. Power Sources* **194**, 1208 (2009).
4. J. R. Miller, R. A. Outlaw, B. C. Holloway, *Science* **329**, 1637 (2010).
5. Y. Gogotsi, P. Simon, *Science* **334**, 917 (2011).
6. J. R. Miller, *Batteries Energy Storage Technol.* **35**, 115 (2012).
7. Capacitor costs were derived by using the largest commercial products available.
8. ECs are sometimes referred to by the product names supercapacitor or ultracapacitor.
9. J. R. Miller *et al.*, *Electrochem. Soc. Interface* **17**, 53 (2008).
10. V. Strong *et al.*, *ACS Nano* **6**, 1395 (2012).

10.1126/science.1219134

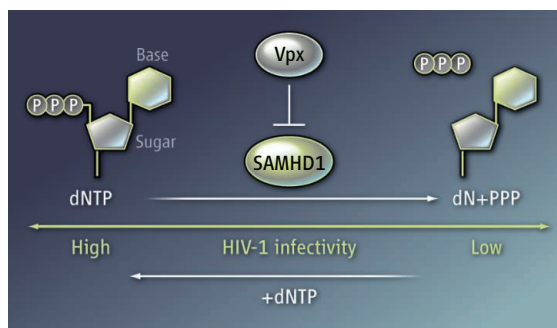
AIDS/HIV

HIV Interplay with SAMHD1

Torsten Schaller, Caroline Goujon, Michael H. Malim

New insights into the complex interplay between human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) and their primate hosts are being gleaned from studies of viral accessory proteins. It is now apparent that these proteins (which are often dispensable for replication in cell culture) frequently antagonize host innate and adaptive immune responses. In several cases, accessory proteins repress specific host cell inhibitors of infection known as restriction factors (1). That the genes encoding restriction factors have been subjected to Darwinian selection pressure suggests the evolutionary importance of their function (2, 3). These general themes have recently been reprised through studies on the Vpx accessory protein of HIV-2/SIV_{smm} and the discovery of its host cell target called sterile alpha motif (SAM) and histidine/aspartic acid (HD) domain-containing protein 1 (SAMHD1).

Cells of myeloid origin (including dendritic cells and monocytes) are often poorly permissive for HIV-1 infection compared to activated CD4⁺ T cells. HIV-1 infectivity can



Restricting HIV infection. SAMHD1 regulates dNTP concentrations and HIV-1 infection in myeloid cells. The viral accessory protein Vpx blocks SAMHD1, targeting it for destruction by proteasomes. PPP, triphosphate

be increased by introduction of the Vpx protein of HIV-2 and some SIV strains (4). Similarly, Vpx-deficient strains of HIV-2/SIV_{smm} are much less infectious in myeloid cells than wild-type viruses. The Vpx-mediated enhancement of infectivity correlates with the increased accumulation of viral cDNAs (4), key intermediates in the replication of retroviruses.

The *vpx* gene most likely originated by recombination or duplication of *vpr*, a related gene in primate lentiviruses whose contribution to HIV-1 replication and pathogenesis

A protein that controls nucleic acid metabolism affects the balance between HIV infection and immune responses.

remains uncertain. Vpx and Vpr engage cellular ubiquitin ligase complexes (which modify proteins, among other activities, for destruction by proteasome), providing the rationale for seeking cellular Vpx/Vpr binding partners as candidate regulators of HIV and SIV replication. Last year, SAMHD1 was defined as a target for Vpx: As predicted, SAMHD1 is degraded by the proteasome in the presence of Vpx, and its depletion from myeloid cells promotes more efficient HIV-1 infection (5, 6).

More recently, *in vitro* enzymatic assays demonstrated that the dimeric SAMHD1 core domain has 2'-deoxyguanosine 5'-triphosphate (dGTP)-stimulated deoxynucleoside triphosphate (dNTP) triphosphohydrolase activity and converts dNTPs to deoxynucleotides (dNs) and triphosphate (7, 8). By showing that Vpx expression or SAMHD1 depletion each increases the amount of dNTPs in macrophages, and that the extracellular addition of dNs to macrophages enhances HIV-1 infection (9), a model has emerged in which SAMHD1 inhibits HIV and SIV infection of myeloid cells by elimi-

nating the dNTP substrates required for viral cDNA production (a step in the genome replication process involving reverse transcription) (see the figure).

The assignment of SAMHD1 as a viral restriction factor is not the first time this protein has been associated with innate immunity. Mutations in *SAMHD1* can cause Aicardi-Goutières syndrome (AGS), a rare autoimmune disease resembling congenital infections, in which excessive production of the cytokine interferon- α (IFN- α) and detrimental immune activation are prominent. Importantly, monocytes of AGS patients that harbor nonfunctional SAMHD1 protein are susceptible to infection by HIV-1 *ex vivo*, whereas cells from healthy donors are not (10).

In addition to *SAMHD1*, mutations in four other genes have been linked to AGS: *TREX1*, *RNAseH2A*, *RNAseH2B*, and *RNAseH2C*. Intriguingly, two of these factors (*TREX1* and *RNAseH2A*) stimulate HIV-1 replication, the opposite of SAMHD1's effect in myeloid cells (11, 12). *TREX1*, a 3'→5' deoxyribonuclease (DNase) involved in removing endogenous retrotransposon-derived cDNA, may also degrade surplus HIV-1 cDNAs thereby averting an IFN response and the induced expression of antiviral genes (11). Elucidating the role of SAMHD1 in dNTP metabolism and viral cDNA synthesis provokes questions regarding the natural functions of SAMHD1, and the molecular pathogenesis of AGS. For instance, do inactivating *SAMHD1* mutations lead to abnormal levels of cDNAs encoded by retroelements (or other templates), as observed in the absence of *TREX1*? If this is the case, is this confined to myeloid and/or postmitotic cells, and is elevated dNTP concentration the underlying mechanism? Importantly, there are AGS patients who do not have mutations in *SAMHD1*, *TREX1*, *RNAseH2A*, *RNAseH2B*, or *RNAseH2C*: This suggests that additional protein(s) acting at the interface of cellular nucleic acid metabolism and innate immunity may affect HIV-1 biology.

Unlike the Vpr proteins of some SIVs (2), HIV-1 Vpr does not antagonize SAMHD1, at least for the strains studied so far. One possibility is that other viral elements may compensate and perform this function. Alternatively, infections of myeloid cells may have a limited role in the spread and pathogenesis of pandemic HIV-1 strains, potentially because these viruses replicate efficiently in CD4⁺ T cells. Indeed, forcing HIV-1 to infect dendritic cells by providing Vpx in *trans* triggers an IFN response (13), perhaps suggesting that SAMHD1 may promote overall replication *in vivo* by perturbing aspects of the human

immune response to HIV-1. Possibly corroborating this notion are observations that HIV-2, whose Vpx protein inhibits SAMHD1, is generally less pathogenic than HIV-1; likewise, SIV_{md1} seems to be a more pathogenic virus than SIV_{md2}, and only SIV_{md2} encodes a Vpx protein that antagonizes SAMHD1. Further analyses will be needed to test these ideas, and it will be interesting to examine the N-, O-, and P-groups of HIV-1 that have not efficiently established themselves in humans. Moreover, nonprimate lentiviruses that preferentially infect myeloid cells, such as maedi-visna or caprine arthritis-encephalitis virus, do not encode obvious Vpx/Vpr proteins: Have these viruses evolved other ways to counteract SAMHD1?

Demonstrating that SAMHD1 inhibits HIV-1 infection at an early step of replication after entry of virus into the cell reinforces the view that this phase of the virus life-cycle is exquisitely vulnerable to suppression by host restriction factors. When operative, the actions of restriction factors, including apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (APOBEC3) proteins, and tripartite motif-containing protein 5 alpha (TRIM5 α), are also manifested as the inability of HIV and SIV to produce functional reverse transcripts

(1). However, in contrast to APOBEC3 and TRIM5 α , where pharmacologic induction of their antiviral functions are desirable, the enhancement of SAMHD1 action could be contraindicated because this restriction factor may impede the elaboration of effective innate and adaptive immunity. On the other hand, there is the exciting prospect that judicious blockade of SAMHD1 (or *TREX1*) may evoke improved immune responses, and that agents with this property could be developed as a new class of antiretroviral drugs.

References

1. M. H. Malim, P. D. Bieniasz, in *HIV: From Biology to Prevention and Treatment*, F. D. Bushman, G. J. Nabel, R. Swanstrom, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, NY, 2012), pp. 119–134.
2. E. S. Lim *et al.*, *Cell Host Microbe* **11**, 194 (2012).
3. N. Laguet *et al.*, *Cell Host Microbe* **11**, 205 (2012).
4. C. Goujon *et al.*, *Retrovirology* **4**, 2 (2007).
5. N. Laguet *et al.*, *Nature* **474**, 654 (2011).
6. K. Hrecka *et al.*, *Nature* **474**, 658 (2011).
7. D. C. Goldstone *et al.*, *Nature* **480**, 379 (2011).
8. R. D. Powell, P. J. Holland, T. Hollis, F. W. Perrino, *J. Biol. Chem.* **286**, 43596 (2011).
9. H. Lahouassa *et al.*, *Nat. Immunol.* **13**, 223 (2012).
10. A. Berger *et al.*, *PLoS Pathog.* **7**, e1002425 (2011).
11. N. Yan, A. D. Regalado-Magdos, B. Stiggelbout, M. A. Lee-Kirsch, J. Lieberman, *Nat. Immunol.* **11**, 1005 (2010).
12. A. Genovesio *et al.*, *J. Biomol. Screen.* **16**, 945 (2011).
13. N. Manel *et al.*, *Nature* **467**, 214 (2010).

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CHEMISTRY

Getting Molecular Electrons into Motion

Markus Gühr

A method based on correlated measurements of electrons and ions shows that electrons of large molecules can be set into motion by strong laser fields.

The valence electrons of a molecule are responsible for making chemical bonds, and their interactions with one another determine whether processes such as electron transfer between molecular groups in light harvesting occur. Electrons are so light that their motion within molecules is described on time scales of attoseconds (1 as = 10⁻¹⁸ s). The promise of attoscience is to use ultrafast laser pulses to excite processes in atoms and molecules that occur on these time scales and thereby probe electron interactions. So far, attosecond time-resolved

experiments have only been accomplished for isolated atoms and very small molecules. On page 1336 of this issue, Boguslavskiy *et al.* (1) now show that electrons of larger and more complex molecules can also be set into attosecond motion through ionization processes induced with strong electric fields generated by laser pulses.

Attosecond time-resolved experiments are done in a perturb-and-observe manner: An initial laser pulse takes one electron out of the molecule (ionization), and a second laser pulse probes the reaction of the remaining electrons to that loss. Boguslavskiy *et al.* concentrate on the ionization step to set the electrons into motion. They used an infrared laser

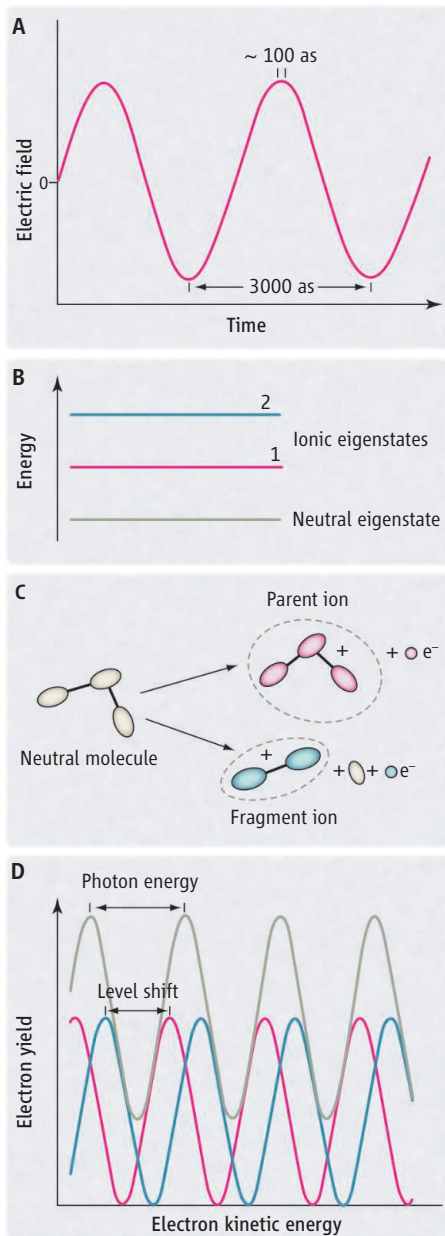
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pulse of 80 fs (80,000 as) duration, consisting of many 3000-as cycles to accomplish repetitive molecular ionization. Panel A of the figure shows the electric field oscillations within the strong infrared laser pulse. The electric field strength in an interval of ~ 100 as around the peak of every cycle can be high enough to tear off a valence electron, a phenomenon called strong-field ionization (SFI).

Because of the laws of quantum mechanics, the short ionization time for SFI is alone not a sufficient condition for really setting the molecular electrons in motion. The electrons in a molecule reside in so-called eigenstates, which have very well-defined energies but no net electronic motion. In order to start electron motion, several eigenstates need to be populated at once. For Boguslavskiy *et al.*, the electrons of the molecular ion just after SFI needed to be in a superposition of at least two eigenstates (panel B). Early work on SFI suggested that only the lowest ionized state could be populated, but for atoms, strong laser fields can indeed populate the superposition of two ionic eigenstates (2–4). For molecules, similar evidence has been obtained only for small molecules (5–8). Larger and more complex molecules should provide interesting results such as flow of charge over large distances in a molecule (9, 10), but so far there has not been any experimental evidence for electronic dynamics.

Boguslavskiy *et al.* now show that SFI can create multiple ionic eigenstates relatively large hydrocarbon molecules. They measured the arrival times of the electrons that escape the molecules in SFI and calculated their velocities and kinetic energies (gray curve in panel D of the figure). The kinetic energy of the electrons has a comb-like modulation with intervals of the laser photon energy between the individual teeth, called an “above threshold” ionization spectrum (11, 12). The absolute energy of the comb is proportional to the energy of the ionic eigenstate after SFI. A molecule left in several eigenstates will give rise to several shifted combs (as shown in panel D of the figure, with red coming from eigenstate 1 and blue emerging from eigenstate 2), but the teeth of the individual combs are too broad and too dense to really be distinguished in the sum over all electrons (black line).

To overcome this difficulty, the authors measured the mass of the ion left behind after SFI separately for every detected electron. Panel C of the figure shows that SFI can either leave the molecule intact (to form the so-called parent ion) or fragment it, leading to the production of a lighter ion and a neutral fragment. Boguslavskiy *et al.* plotted the



comb of the sorted electrons belonging to parent ions (red) and separately for breakup into certain masses (only one of these fragment channels is depicted in blue). The sorted combs showed different absolute energies, which is direct evidence for the excitation of different eigenstates in the ion and thus for electronic dynamics.

The electron comb belonging to the parent ion has the energy of the lowest ionic eigenstate. The combs for ions with smaller masses are associated with higher-lying excited ionic eigenstates. It might be argued that one could just measure the masses of the ions left behind instead of doing the complicated correlation with electrons. However, Boguslavskiy *et al.* caution against this approach, because, under certain conditions,

Strong-field ionization of large molecules. (A)

Boguslavskiy *et al.* studied the strong-field ionization of large molecules in the oscillating electric field of a laser consisting of many periods of about 3000-as duration. The ionization (emission of an electron) only occurred at the highest fields within ~ 100 as in each period. (B) A superposition of several ionic eigenstates (here just two states, 1 and 2, are depicted) above the neutral state creates the conditions for electron motion in a quantum-mechanical sense. (C) Boguslavskiy *et al.* measured the kinetic energy of the electrons in correlation with either parent or fragment ions. (D) The kinetic energy of electrons emitted in the strong laser field has a comb-like structure modulated by the photon energy. The kinetic energies of electrons belonging to the parent (red) and one fragment ion (blue) channel exhibit a shift, indicating that the electrons belong to different ionic eigenstates. Thus, two eigenstates are created by SFI and the remaining electrons on the ion will move.

the laser field can populate higher eigenstates many attoseconds after the ionization occurs.

The scheme of Boguslavskiy *et al.* should allow for measurements of ion momenta and angular distributions correlated with ionization and provide more detailed information on the eigenstate in which the ion is left after SFI. The next major step would be to observe the electronic motion initiated by the strong laser field using an attosecond probe pulse (4) and analyze it. The dynamical features of multielectron interactions—a topic impossible to treat accurately in quantum simulations—would serve as a gauge for theory.

Nature uses femtosecond (1000 as) processes for efficient molecular charge and energy transfer in light harvesting and vision. Simulations suggest that attosecond ionization triggers electronic motion from one to the other end of a large molecule on even faster attosecond time scales (9, 10). With electrons traveling at their ultimate speed in molecules, we might thus reach the ultimate efficiencies in molecular charge and energy transport.

References

1. A. E. Boguslavskiy *et al.*, *Science* **335**, 1336 (2012).
2. L. Young *et al.*, *Phys. Rev. Lett.* **97**, 083601 (2006).
3. Z.-H. Loh *et al.*, *Phys. Rev. Lett.* **98**, 143601 (2007).
4. E. Goulielmakis *et al.*, *Nature* **466**, 739 (2010).
5. B. K. McFarland, J. P. Farrell, P. H. Bucksbaum, M. Gühr, *Science* **322**, 1232 (2008).
6. W. Li *et al.*, *Science* **322**, 1207 (2008).
7. O. Smirnova *et al.*, *Nature* **460**, 972 (2009).
8. J. P. Farrell *et al.*, *Phys. Rev. Lett.* **107**, 083001 (2011).
9. S. Lünemann, A. I. Kuleff, L. S. Cederbaum, *Chem. Phys. Lett.* **450**, 232 (2008).
10. F. Remacle, R. D. Levine, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 6793 (2006).
11. P. Agostini, F. Fabre, G. Mainfray, G. Petite, N. Rahman, *Phys. Rev. Lett.* **42**, 1127 (1979).
12. L. F. DiMauro, P. Agostini, *Adv. Mol. Opt. Phys.* **35**, 79 (1995).

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RETROSPECTIVE

Norton Zinder (1928–2012)

Harvey Lodish¹ and Nina Fedoroff²

Norton Zinder, a founder and leader of the then nascent field of molecular biology half a century ago, passed away on the 3rd of February in the Bronx, New York. He was a Professor at the Rockefeller University, where we were privileged to have been his graduate students and experienced how insightful mentorship and a fertile environment can inspire young students to do great science.

Norton was born in New York in 1928 and graduated at the age of 18 from Columbia University, going on to do his doctoral research with the future Nobel laureate Joshua Lederberg at the University of Wisconsin. Lederberg had discovered that certain strains of the bacterium *Escherichia coli* could transfer genes by direct cell-to-cell contact. Norton soon found that genetic exchanges also occur in *Salmonella* strains but that they were mediated by filterable particles—that is, bacterial viruses (bacteriophages). This was the discovery of what came to be called transduction, and it presaged later findings that mutant cellular genes can be incorporated into retroviruses to transform the cells they subsequently infect.

Norton moved to Rockefeller University in 1952 and stayed there for the rest of his career. Although Norton viewed the discovery of transduction as his signal achievement, many substantial contributions followed his isolation in 1961 of the first RNA bacteriophage, f2, and the first single-stranded DNA bacteriophage, f1. These included evidence that f2 RNA supports the synthesis of phage coat protein in a cell-free extract, the first demonstration that a naturally occurring messenger RNA could generate an authentic protein. This was quickly followed by the discovery that the in vitro-synthesized



coat protein had an extra *N*-formyl-methionine at its amino terminus. Together with a contemporaneous paper from James Watson's laboratory showing the existence of *N*-formyl-methionyl-transfer RNA, this discovery established the mechanism of polypeptide chain initiation, completing elucidation of the genetic code.

Genetic and molecular analysis of f2 mutants subsequently identified the three proteins encoded by the phage RNA, includ-

ing a subunit of its RNA-dependent RNA polymerase, and defined its mechanism of replication as proceeding through the synthesis of a complementary “minus” strand, which in turn serves as the template for the synthesis of the phage “plus” strands packaged into viral particles. This conclusion was controversial at the time (1966): Sol Spiegelman's lab had reported that plus strands directly template synthesis of more plus strands by a “non-Watson-Crick” replication mechanism.

Norton's lab was later instrumental in defining the cleavage mechanism of type I restriction endonucleases, which bind to a specific DNA sequence, then move thousands of nucleotides away before cleaving the DNA. These were found to be multifunctional proteins with both restriction and methylation activities.

Norton took a leadership role in the controversies surrounding the development of recombinant DNA technology and, later, the sequencing of the human genome. He was a signatory of the famous 1974 Berg letter articulating the potential biohazards of recombinant DNA molecules and an organizer of the historic 1975 Asilomar Conference that called for a moratorium on recombinant DNA research. These led to the establishment of the United States National Institutes of Health (NIH) Recombinant DNA Advisory Committee and gave rise to a complex set of containment guidelines for recombinant DNA research. As experience with recombinant DNA accumulated

The seminal work of this geneticist and microbiologist on bacteriophage contributed importantly to our understanding of fundamental genetic processes.

without evidence of special hazards, Norton came to believe that the guidelines should be completely abolished, except for applications to plants, which he thought merited a cautious approach because of their ability to reproduce through seeds.

In the late 1980s, Norton played a crucial role, together with James Watson, in initiating the Human Genome Project, itself controversial at the time. Many biomedical scientists feared “big science” and were concerned that the NIH would reduce support for investigator-initiated research grants, the R01s. Zinder and others persisted, speaking publicly and testifying before multiple congressional committees about the importance of sequencing the entire human genome, not just the protein-coding segments. It was already known that protein-coding sequences comprise a small fraction of the DNA and the rest had been labeled “junk.” Today, we increasingly recognize the importance of noncoding RNAs and regulatory regions of the genome, as well as the importance of transposons as drivers of evolution. Norton foresaw what many others could not, and he argued forcefully, both in public and in private, for sequencing all of the genome. He is also credited with mediating the 2000 “truce” between the public genome-sequencing efforts and Craig Venter's private sequencing project.

Norton Zinder was the recipient of many honors and awards, including the U.S. National Academy of Science's Award in Microbiology in 1966. He was elected to the American Academy of Arts and Sciences in 1968 and the U.S. National Academy of Sciences in 1969. Norton's late wife Marilyn helped to maintain a friendly and interactive atmosphere in the lab with frequent warm and informal dinners at their house in Queens and later in their Manhattan apartment. Marilyn's continued support was essential to Norton's achievements, both in his scientific discoveries and as spokesman for the responsibilities of scientists. He derived much pleasure from the successes of both his scientific offspring—his former students—and of his two sons. His influence extended well beyond his scientific contributions to shaping policy on important issues at the interface between science and society.

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Human Evolution Out of Africa: The Role of Refugia and Climate Change

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Although an African origin of the modern human species is generally accepted, the evolutionary processes involved in the speciation, geographical spread, and eventual extinction of archaic humans outside of Africa are much debated. An additional complexity has been the recent evidence of limited interbreeding between modern humans and the Neandertals and Denisovans. Modern human migrations and interactions began during the buildup to the Last Glacial Maximum, starting about 100,000 years ago. By examining the history of other organisms through glacial cycles, valuable models for evolutionary biogeography can be formulated. According to one such model, the adoption of a new refugium by a subgroup of a species may lead to important evolutionary changes.

Modern humans are thought to have evolved in Africa more than 200,000 years ago. By about 20,000 years ago, they had expanded to all continents except the Americas and Antarctica and had essentially replaced all archaic human species. The most familiar of these, the Neandertals, was spread across western Eurasia, but recent evidence indicates that other human forms were also present, such as the Denisovans in Siberia and *Homo floresiensis* on the Indonesian island of Flores. DNA evidence now implies that modern humans interbred with at least some of these native species. Migration and replacement occurred as climates fluctuated toward the peak of the last Ice Age. The human patterns emerging from these new data have led to debate regarding the roles of climate oscillations and the resultant refugia in the formation of these patterns.

We suggest that recent studies of changes in the biogeography of other organisms, and of their constituent populations through the Pleistocene (1–3), can provide a potential model for human evolution outside Africa. Phylogeographic (genetic biogeographical) studies of various extant organisms, paleontological studies, and ancient DNA (aDNA) techniques are beginning to reveal the important role of population contraction into refugial areas in driving the evolution of distinct lineages of species, and are leading to the implication of refugia as areas of endemism for new populations and species (2, 4–6). Additionally, aDNA analyses are showing that extinction below the species level was far more prevalent than formerly realized (7). Here, we integrate these studies with recent evidence of human migration and interactions to assess some of the factors

influencing the unique emergence of one globally distributed species of human by the end of the Late Pleistocene.

What Was the Role of Quaternary Refugia in the Evolution of Organisms in General?

The idea that species' geographical ranges changed during glacial cycles dates back as far as Darwin (8). However, it was in the 1950s that the term refugia was first used by palynologists to describe the contracted ranges of plants during the last glacial in Canada (9). Since that time it has been applied in many different environmental contexts, including tropical forests in Amazonia (10). The definition we use here is in essence that of Hewitt (4), in which a refugium is an area where a particular species survived for an entire glacial-interglacial cycle. This will generally be the smallest space occupied by the fewest numbers of individuals over time (2, 4). A species' adaptations and tolerances will influence the time and place at which the refugium occurred during a glacial cycle (6). Note, however, that a recent paper advocated that the term be dropped in favor of the alternative concept of bottlenecks (11).

In Eurasia, the concept of the refugium was originally applied to temperate-adapted taxa whose populations contracted during glacial periods. In Europe, such taxa were thought to have refugia in the southern peninsulas (Iberia, Italy, and the Balkans) as well as in the east (2, 4, 12). This conclusion was based on phylogeographical studies of animals and plants [the common meadow grasshopper (*Chorthippus parallelus*), hedgehogs (*Erinaceus* sp.), brown bear (*Ursus arctos*), and oak trees (*Quercus* sp.)], showing that genetically distinct populations of these species were distributed across Europe, but with greater continuity between populations in different southern regions and areas to the north. The inference that refugia lay to the south came from the influence of palynological studies [e.g., (13, 14)], where trees had been reconstructed as expanding out of southern

Europe as climate warmed during the Holocene. The phylogeographic patterns were in turn used to infer that populations expanded from these putative southern refugia (4). In addition to southern refugia, there seem to have been cryptic northern refugia at higher latitudes, where temperate taxa persisted through the last glacial (5, 15, 16). In contrast, the ranges of colder-adapted taxa, and especially arctic-adapted ones, decreased during interglacials such as the Holocene. For example, the ranges and abundances of species such as the collared lemming (*Dicrostonyx torquatus*) and reindeer (*Rangifer tarandus*), whose distributions are limited to the north today, and in some cases also to southern mountain ranges [such as the mountain avens (*Dryas octopetala*) in the Alps], expanded during the last glacial (6). Similarly, continental-adapted taxa [e.g., ground squirrels (*Citellus* sp.) and saiga (*Saiga tatarica*)] had expanded ranges during glacial times, whereas they are in refugia today in central Eurasia (6). Many oceanic taxa are widespread today but were in refugia during glacial times. A further scenario is that offered by the analysis of aDNA of brown bear in Europe (17), which has suggested that in some instances, species had a single larger southern refugium encompassing all the peninsular refugia, but with limited gene flow between peninsulas.

Evidence shows that local extinctions can occur when a species' range is contracting (18), although it remains unknown whether this is a general phenomenon. If this does occur, then long-term refugia will generally lie where terminal populations eventually become extinct (19). The expansion and contraction of a species' range can also influence its evolution. Populations in refugia will tend to differentiate from other refugial populations through drift and natural selection in response to the specific conditions encountered; ecological variation between separate refugia could help to reinforce differences between the isolated populations (2, 4, 20). The alternative view is that differentiated populations may mix and merge again when ranges expand during more favorable conditions (21, 22).

Phylogeographic studies [e.g., (2)] have shown that when isolated populations of temperate taxa reemerged from southern refugia in Europe during interglacials, hybrid zones were formed, but a single population did not generally reform completely. The results suggest that these refugia may have been areas of endemism for temperate taxa (2, 5). An example of this process is the evolution of two hedgehog species in Europe, the western hedgehog (*Erinaceus europaeus*) and the eastern hedgehog (*Erinaceus concolor*), which appear to have expanded out of Spain and Italy and out of the Balkans, respectively (23). Evolution may also take place in cryptic refugia: For example, the polar bear (*Ursus maritimus*) is known from mitochondrial DNA (mtDNA) studies to be an arctic-adapted brown bear (24). It likely evolved when brown bears were isolated in a cryptic

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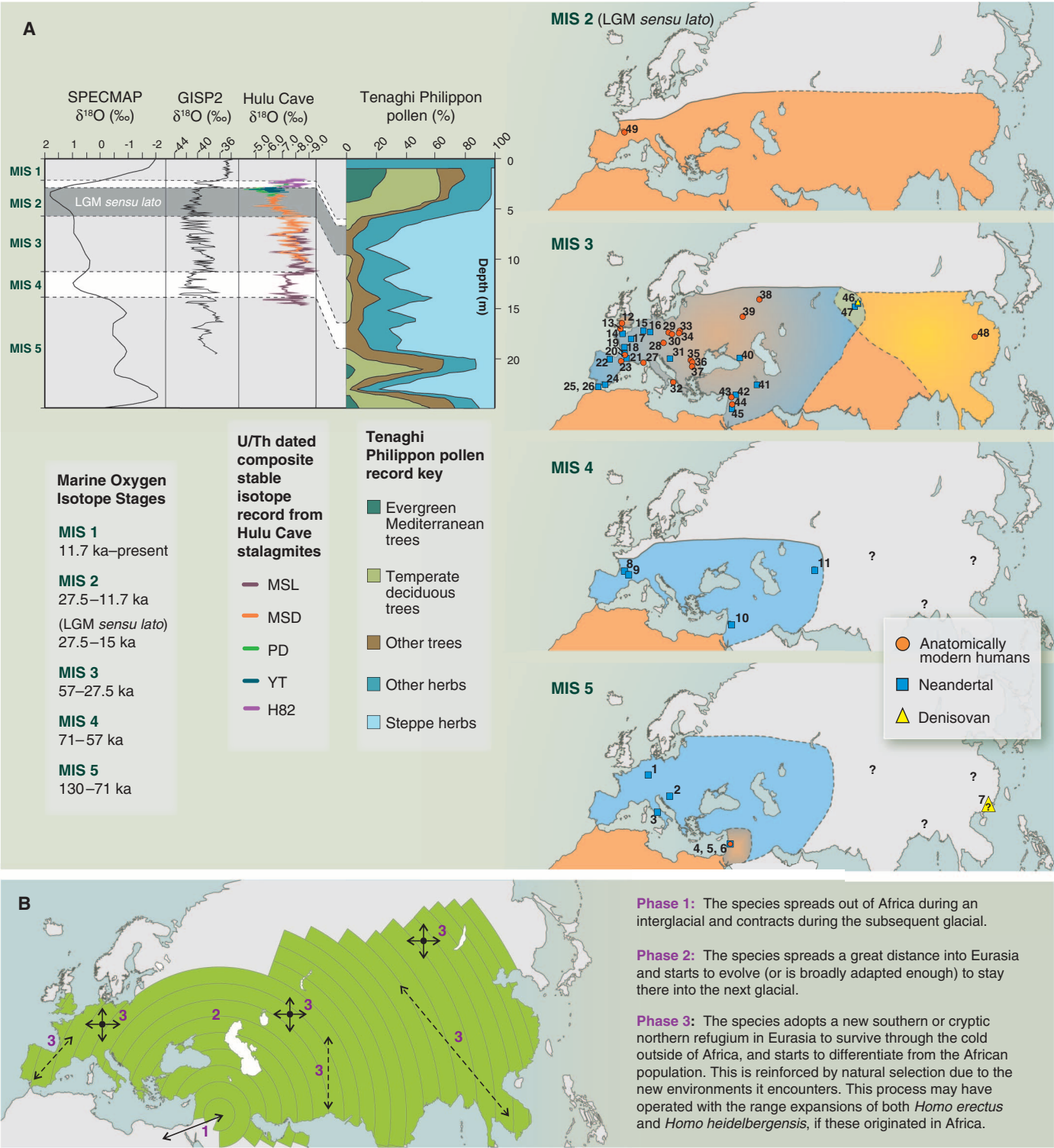


Fig. 1. Patterns and process of human distribution in Eurasia between 130,000 and 15,000 years ago in relation to climate change. **(A)** Long-term climate change curves for the Late Pleistocene, 130,000 years ago (130 ka) to the present. Included are SPECMAP chronology, GISP data, the Hulu Cave stalagmite curve, and the overview pollen record from Tenaghi Philippon (92–95). Also shown are maps of Eurasia with the distributions of selected (well-dated) sites with reliably identified human remains (Neandertals, AMH, and Denisovans) in different broad climatic episodes [marine oxygen isotope stages (MIS) 5, 4, 3, and 2, although the younger limit of MIS 2 is taken here to be the start of the first Late Glacial interstadial at ~15,000 years ago]. Table S1 is a key to the numbered

sites. The maps also include a projected maximum distribution of humans (Neandertals, AMH, and Denisovans) based on archaeology. Our confidence in the limits of these distributions varies, as indicated by the solid (more confident) and dashed (less confident) lines. **(B)** Diagram showing the three-phase process whereby human populations expanded their range out of Africa, adopted refugia in Eurasia, and eventually differentiated into distinct populations and species. These refugial areas may also have been operating during the shorter stadials. Alternatively, the human populations outside of Africa may have occupied a broad, continuous range across Eurasia during glacials, invoking a parapatric or even a sympatric evolutionary mode.

coastal northern refugium, whereupon selection favored adaptations to arctic maritime conditions (6). aDNA analyses of Quaternary fossils have supported this idea and indicated that the origin may have occurred in an area such as Ireland (25).

Ancient biomolecules have also shown that past mammals often had greater genetic variability in the Late Pleistocene than do their extant counterparts (7), suggesting that many mammals [e.g., brown bear, bison (*Bison bison*), musk ox (*Ovibos moschatus*)] underwent population bottlenecks (26–28). Such high variability is consistent with the presence of some extinct clades in the fossil record that have been interpreted morphologically as different species or subspecies, such as the cave lion (29).

Although disagreements exist about details, it is generally thought that the uniqueness of species (or populations of those species) and their specific adaptations guide the location, size, and timing of refugia for a given organism (6, 11). This perspective can be applied to different populations of the genus *Homo* through time to gain an insight into how climate change, or other natural events, affected their range and abundance. In turn, a number of predictions or hypotheses can be derived about the evolution of those human populations.

What Was the Biogeographical Pattern of Later Pleistocene Humans Outside of Africa?

Our view of the human inhabitants of Eurasia during the past 130,000 years (Fig. 1) has changed recently. There is consensus that Neandertals (*Homo neanderthalensis*) occupied the west of the continent for more than 200,000 years, although our knowledge of Neandertals in eastern Eurasia is much more limited at present. By at least 40,000 years ago they had been joined by anatomically modern humans (AMH; *Homo sapiens*) (30–32). Neandertals apparently became extinct shortly afterward (33, 34). This extinction was apparently diachronous across Europe, starting in the north and culminating in their final demise to the south (35). The last populations were evidently in southern refugia such as Iberia (36), the Balkans (37), and possibly the Levant (38). The contraction may have paused in areas such as Belgium until around 36,000 years ago (39) because southern Belgium was a cryptic northern refugium for a number of species at this time (Fig. 1) (15). The contraction of the range of Neandertals was roughly coeval with the arrival of AMH, although it is possible that the Neandertals had already disappeared from northern areas by the time AMH arrived there (30). However, many have directly linked the arrival of AMH with the disappearance of Neandertals and have cited competition between the two human species as a likely cause [e.g., (33, 40)]. Others have attributed their extinction to a combination of climatic factors and competition with AMH (41). A dominant role for climate in Neandertal extinction also has its supporters (42, 43). Earth's

climate became cooler after ~100,000 years ago, and ice sheets expanded to the Last Glacial Maximum (LGM) ~20,000 years ago. However, the overall cooling involved several abrupt episodes, and recent focus has been on the extreme cold of Heinrich event H4 (~39,000 years ago) or H5 (~48,000 years ago) as primary drivers of Neandertal extinction (30, 38, 40, 44).

Although Neandertals appear from existing data to have finally died out in southern Europe, we now know from aDNA results that their geographical range extended to Siberia at times (Fig. 1) (45, 46). It is not known whether the nearest refugium for these eastern populations was immediately south of Siberia or was farther west, although it seems unlikely that Siberia itself was a refugium, given that the Neandertals retreated south as climate cooled toward the LGM in Europe (35). Neandertal fossils in Iraq and the Levant (Fig. 1) suggest that refugia existed in such areas, in addition to western refugia such as Iberia. However, the present evidence does not clearly indicate that these populations represented different Neandertal clades, which may imply that if eastern refugia existed, they had not been operating as such for long [(45), but compare (47)].

The most unexpected result from the eastern localities was that the aDNA data—both mitochondrial and nuclear—suggested that another distinct human population, the Denisovans, lived in Siberia at the same time as Neandertals, near the time that the latter were becoming extinct. On the basis of their mtDNA, the Denisovans were originally described as different from both AMH and Neandertals (48), but they are now considered to be a sister group of Neandertals by virtue of a younger coalescence date for their nuclear DNA (49). Moreover, the three Denisovan mtDNA sequences so far obtained (from a single site) are already more diverse than all those known from the Neandertals (46). This genetic diversity in Pleistocene humans, together with the additional variation 100,000 years ago suggested by the mtDNA of the Neandertal fossil from Sladina, in Belgium (50), mirrors the genetic diversity seen in other mammals during the Pleistocene (7). Although morphological information on the Denisovans is sparse relative to the genomic data, fossils from China (e.g., Dali, Maba) and India (Narmada) might represent this Asian lineage. These have variously been regarded as related to *Homo erectus*, *Homo heidelbergensis*, Neandertals, or archaic *H. sapiens* (51).

Before the emergence of the Neandertals, Eurasia was occupied by *H. erectus*, a species that evolved perhaps 2 million years ago and is seen in Dmanisi, Republic of Georgia, by at least 1.8 million years ago (52). Several other *Homo* species followed, notably *H. heidelbergensis*, which is arguably found throughout Eurasia from about 600,000 to 400,000 years ago (51). The timing of the last *H. erectus* occurrence in continental Eurasia is uncertain, and it may well be that the species disappeared during the

Middle Pleistocene for reasons that are currently unknown. It had been thought to have survived until the Late Pleistocene at Ngandong in Java (Indonesia); however, the date of these fossils is not well established (53). The enigmatic *H. floresiensis* (54, 55), which is dated from about 95,000 to 17,000 years ago, is of uncertain affinity and is an isolated insular find. Thus, we exclude these two species from the discussion of continental refugia; unless aDNA can be retrieved from *H. erectus* and *H. floresiensis*, the precise relation between each of these species and the Neandertal, Denisovan, and AMH groups may remain uncertain. It is likely that, as with Neandertals, the extinction of other archaic humans took place in their respective refugial areas, as this is the direction toward which the last populations will have contracted in geographical range and numbers of individuals.

The earliest known AMH outside of Africa are from the Levant, dated between 90,000 and 120,000 years ago (Fig. 1A) (56). It seems likely that this was a population expansion that contracted again toward Africa or Arabia during a subsequent cold or arid event. The next expansion of AMH outside of Africa was ~60,000 years ago, when they appear to have spread into western Asia (Fig. 1A) (57). This population expansion led to their dispersal into Australasia by at least 45,000 years ago (58).

The arrival of AMH in Europe by at least 40,000 years ago (31, 32, 59) represented an expansion of their geographical range from the east, although the geographical origin of these dispersals is not precisely clear (57). After ~26,000 years ago, AMH, now the sole occupant of Europe, also retreated south and became locally extinct in northwestern Europe (Fig. 1A). This occurred as ice sheets advanced and ice-free northern environments became impoverished in terms of carrying capacity. The contraction of the AMH geographic range appears to have been a two-stage process in northwestern Europe, as there is evidence that areas such as Belgium were vacated by AMH making Aurignacian industries, only to be repopulated some time later by a population making the Gravettian stone tool industry, as interstadial conditions temporarily returned just before the LGM (60). The Gravettian industry eventually disappeared in turn, about 23,000 years ago, after which northwestern Europe lacks evidence of human occupation for about 8000 years until the area was recolonized from one or more southern and/or eastern refugia in the Late Glacial ~15,000 years ago (60). Indeed, it was as a consequence of a similar Late Glacial spread north that AMH crossed the Bering Straits and dispersed into North and South America (61).

How Did the Evolution of Archaic Out-of-Africa Humans Take Place?

When a lineage adopts a new refugial area and survives for a number of Milankovitch cycles, expanding from and contracting into that new

refugium instead of its original refugium, it is destined to evolve into a distinct population. Given enough time in isolation, it will become a new species (Fig. 1B) (4–6). Because a new refugium is unlikely to have the same flora, fauna, and ecology as the lineage's original refugium, it exerts selective pressure to adapt and diverge (2, 4, 20). The potential role of ecological adaptation could theoretically even lead to the evolution of two sympatric human species in the same refugium, particularly if heterochrony is involved (62). Therefore, when the initial expansion of archaic *Homo* out of Africa during an interglacial (52, 63) eventually suffered range contraction in the face of climatic deterioration, it was either going to go extinct locally or, because it was sufficiently broadly adapted, survive in an out-of-Africa refugium (Fig. 1B).

It may well be that a broadly adapted taxon such as *Homo* (64) was particularly well disposed to dispersals and eventual differentiation. This appears to have happened to the likely ancestors of Neandertals. *H. heidelbergensis* expanded across Eurasia around 600,000 years ago and persisted there through entire ice age cycles, where it would have become adapted to more northern environmental conditions (Fig. 1B) (65). It apparently retreated into a refugium during cold stages, given the lack of archaeological evidence from such periods in Britain (66). This would have led to a phylogenetic split from the African conspecifics, whose refugial leading edge lay in Africa, or maximally in the Levant region. The eventual result was the distinct human species known as the Neandertals (*H. neanderthalensis*). Thus, the adoption of a new refugium by an expanded part of a population is the mechanism that often leads to phylogenetic speciation within continents.

Neandertals have been described as a cold-adapted, even hyperarctic-adapted, human species (66, 67). Several of their traits, such as relatively short limbs, high body mass, and enlarged sinus cavities, have been interpreted as related to temperature regulation (68, 69). A recent alternative view is that the limb differences are more likely to be related to locomotory adaptations, whereas Neandertal mid-facial shape reflects genetic drift (43, 70). Although these alternative explanations may be valid, there are grounds to infer that Neandertals were cold-adapted to an extent, and certainly more so than were their African ancestors (71). Furthermore, they were probably better physically adapted to cold conditions than the succeeding AMH populations, who instead possessed additional behavioral adaptations that allowed them to cope well with the Late Pleistocene Eurasian environment (71). An interesting contrast here is the evolution of the polar bear (*Ursus maritimus*) when compared with the closely related brown bear (*U. arctos*). Polar bears were and are in refugia during interglacials and were probably more widely distributed during glacial stages—the opposite situation to the Neandertals and many other species.

The age of the most recent common ancestor of Neandertals and AMH could restrict the possible explanations for how and when the various evolutionary events took place. Genetic and craniometric findings suggest that this divergence occurred ~350,000 years ago, but with assumption-based uncertainties such as calibration and generation time (72, 73). The uncertainty is too great to determine whether the divergence took place during a glacial or an interglacial, although the biogeography of other organisms suggests that it was initiated during an interglacial and consolidated during a subsequent cold stage. *H. heidelbergensis*, the presumed ancestor of both *H. sapiens* and *H. neanderthalensis*, is most likely to have spread from Africa or western Asia during an interglacial, when its population was expanding. This expansion ultimately led to the hypothesized divergence between the population of *H. heidelbergensis* that remained in Africa and evolved into *H. sapiens* and the population that spread in Eurasia to eventually evolve into Neandertals (58).

Implications of Pleistocene Interbreeding Between Eurasian Humans

Paleogenomic data imply that there was some interbreeding between Neandertals and AMH, as well as between Denisovans and AMH, during the Late Pleistocene in Eurasia (49, 74). The present-day traces of archaic human introgression are, however, not localized to the same regions as the aDNA sources. Although such interbreeding may undermine the biological species concept as applied to fossil human groups, recent research has shown that hybridization is not uncommon in the wild today between closely related species (75), and it has been recorded in an increasing number of higher vertebrates (76) and primates (77, 78).

Two biogeographic scenarios may lead to interbreeding between populations of organisms in general. In one scenario (4, 79), two populations may meet during their expansive population phases and form hybrid zones. The results of this process show up in Europe as a genetic pattern where longitudinal areas occur from north to south, apparently caused by the spread of the distinct populations from different southern refugia. In the other scenario, species hybridize during periods of environmental disturbance (76) or if the species are rare, such as has been documented for Darwin's finches (80). So two distinct climatic and biogeographic phases (glacial refugial and interglacial expansive) could have led to the hybridization. It may be that Neandertals and AMH interbred when AMH were expanding out of Africa, causing range overlap with Neandertals. This is likely to have been an interstadial expansion that brought AMH out of Africa and into the Levant (44), where interbreeding with the contracted-range Neandertals, albeit during an interstadial, may have occurred. Given that a modern European genome has no higher levels of introgression than a Chinese or a Melanesian

one, the inferred introgression of Neandertal DNA may have happened before those populations diversified but after the notional separation from sub-Saharan Africans, suggesting a likely time of ~60,000 years ago. Alternatively, the consistently small amount of Neandertal introgression may instead be a measure of the ineffectiveness of hybridization in separate interbreeding events, reflecting intrinsic limits on the process caused by biological, social, or demographic factors (81).

Our knowledge of the Denisovans is too limited to say much about the location and timing of the hypothesized introgression with AMH, but given the geographically limited impact—in Australasians and neighboring populations only (49, 82, 83)—it most likely occurred as modern humans spread eastward, after 60,000 years ago but before the arrival of AMH in Australasia (~50,000 years ago). It possibly took place during the refugial contraction phase of the Denisovans during a colder stadial, as AMH were spreading toward Australasia. Also, because there is evidence that grasslands spread south within south-east Asia into the subtropical region during cold conditions (84), the Denisovan range may have moved south at that time (Fig. 1A).

Given the limits of aDNA preservation, these southerly locations unfortunately may never provide genetic evidence of hybridization events from their fossil record (85). Recent evidence of introgression also appears in modern African populations, such as the San and Biaka pygmies, who may also harbor archaic human DNA acquired as recently as 35,000 years ago (86). Physical evidence of such introgression may come from Later Stone Age fossils showing archaic features, such as at Ishango, Congo (87), and Iwo Eleru, Nigeria (88).

Outlook

A number of questions concerning the evolution of humans outside of Africa remain unanswered. Many of the unknowns involve the pattern of genotypes among archaic humans and ancient and living AMH. Until recently this information was completely unavailable, but as the field of paleogenomics develops, it would be valuable to know how the different archaic fossils relate to each other genetically as well as morphologically. It is intriguing that the Denisovans were evidently more genetically variable than the Neandertals (46), and thus there was apparently more human genetic variation overall in the east of Eurasia during the Pleistocene than in the west. Further findings in this area may reopen the question of where the Neandertals evolved—was it in southern Europe or in Asia? Whatever the case, it may be that the ancestors of Neandertals and Denisovans (presumably *H. heidelbergensis*) went eastward before radiating, resulting eventually in the two Late Pleistocene archaic populations. The distribution of the Neandertals during the cold of MIS 4 and AMH during the LGM *sensu lato* (27,500 to 15,000 years ago) could

suggest that both human species had cryptic northern refugia to the east of Europe, unless they were more continuously distributed (Fig. 1).

To date, only about 20 Neandertals and far fewer fossil AMH have yielded aDNA (89), and the Denisovans are barely represented in the fossil record. It is possible that the Denisovans lived farther west, at least at times, and have not yet been recognized. Additional, complete, Denisovan fossils would help to establish their morphological differences with respect to Neandertals and may clarify how Neandertals and Denisovans relate to *H. heidelbergensis*, *H. antecessor*, and Asian *H. erectus*. However, the distinct but overlapping geographic ranges of these various individualistic human populations imply that they possessed different adaptations.

The phenotypic effect of the introgression of archaic humans with AMH is one current research direction. A recent study found that living *H. sapiens* in Europe and Asia may have acquired a part of their immune system from archaic humans (90). This hypothesis needs confirmation, but it does prompt questions as to the effects of this interbreeding.

Many researchers have used archaeological industries as proxies for Neandertals and AMH [e.g., see (35)]. Now that we have evidence of another distinct population in the Late Pleistocene as well as hybridization between past populations, we need to consider how this affects interpretations of the archaeological industries. The cultural evidence from Denisova needs further clarification, as the layers containing the fossils also contain elements of both Middle and Upper Paleolithic technologies (46). Furthermore, as AMH and Neandertals both made “Middle Paleolithic” and “Upper Paleolithic” stone tool industries at different times and places, the reality may have been even more complex. Contact between populations may well have extended beyond exchanges of genetic material to the transfer of behavior and technology, so even greater caution may be necessary when using behavioral markers as proxies for human species or populations.

Note added in proof: Cryptic northern refugia (15) at higher latitudes have now been confirmed for boreal species (91).

References and Notes

1. J. Aise, *Phylogeography: The History and Formation of Species* (Harvard Univ. Press, Cambridge, MA, 2000).
2. G. Hewitt, *Nature* **405**, 907 (2000).
3. J. Provan, K. D. Bennett, *Trends Ecol. Evol.* **23**, 564 (2008).
4. G. Hewitt, *Biol. J. Linn. Soc.* **58**, 247 (1996).
5. D. T. Bilton et al., *Proc. R. Soc. B* **265**, 1219 (1998).
6. J. R. Stewart, A. M. Lister, I. Barnes, L. Dalén, *Proc. R. Soc. B* **277**, 661 (2010).
7. M. Hofreiter, I. Barnes, *BMC Biol.* **8**, 46 (2010).
8. C. Darwin, *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life* (John Murray, London, 1856).
9. C. J. Heusser, *Can. J. Bot.* **33**, 429 (1955).
10. J. Haffer, *Science* **165**, 131 (1969).
11. K. D. Bennett, J. Provan, *Quat. Sci. Rev.* **27**, 2449 (2008).
12. P. Taberlet, L. Fumagalli, A. G. Wust-Saucy, J. F. Cosson, *Mol. Ecol.* **7**, 453 (1998).
13. B. Huntley, H. J. B. Birks, *An Atlas of Past and Present Pollen Maps of Europe, 0–13,000 Years Ago* (Cambridge Univ. Press, Cambridge, 1983).
14. K. D. Bennett, P. C. Tzedakis, K. J. Willis, *J. Biogeogr.* **18**, 103 (1991).
15. J. R. Stewart, A. M. Lister, *Trends Ecol. Evol.* **16**, 608 (2001).
16. S. A. Bhagwat, K. J. Willis, *J. Biogeogr.* **35**, 464 (2008).
17. C. E. Valdiosera et al., *Mol. Ecol.* **16**, 5140 (2007).
18. L. Dalén et al., *Proc. Natl. Acad. Sci. U.S.A.* **104**, 6726 (2007).
19. A. M. Lister, A. J. Stuart, C. R. Geosci. **340**, 615 (2008).
20. J. R. Stewart, *J. Evol. Biol.* **22**, 2363 (2009).
21. G. R. Coope, in *Diversity of Insect Faunas*, L. A. Mound, N. Waloff, Eds. (Blackwell Science, London, 1978), pp. 176–187.
22. K. D. Bennett, *Evolution and Ecology: The Pace of Life* (Cambridge Univ. Press, Cambridge, 1997).
23. F. Santucci, B. C. Emerson, G. M. Hewitt, *Mol. Ecol.* **7**, 1163 (1998).
24. S. L. Talbot, G. F. Shields, *Mol. Phylogenet. Evol.* **5**, 477 (1996).
25. C. J. Edwards et al., *Curr. Biol.* **21**, 1251 (2011).
26. I. Barnes, P. Matheus, B. Shapiro, D. Jensen, A. Cooper, *Science* **295**, 2267 (2002).
27. B. Shapiro et al., *Science* **306**, 1561 (2004).
28. R. D. E. MacPhee, A. N. Tikhonov, D. Mol, A. D. Greenwood, *BMC Evol. Biol.* **5**, 49 (2005).
29. J. Burger et al., *Mol. Phylogenet. Evol.* **30**, 841 (2004).
30. U. C. Müller et al., *Quat. Sci. Rev.* **30**, 273 (2011).
31. T. Higham et al., *Nature* **479**, 521 (2011).
32. S. Benazzi et al., *Nature* **479**, 525 (2011).
33. P. Mellars, *Nature* **439**, 931 (2006).
34. R. Pinhasi, T. F. Higham, L. V. Golovanova, V. B. Doronichev, *Proc. Natl. Acad. Sci. U.S.A.* **108**, 8611 (2011).
35. T. H. Van Andel, W. Davies, Eds., *Neanderthals and Modern Humans in the European Landscape During the Last Glaciation, 60,000 to 20,000 Years Ago: Archaeological Results of the Stage Three Project* (McDonald Institute for Archaeological Research, Cambridge, 2003).
36. C. Finlayson et al., *Nature* **443**, 850 (2006).
37. T. Higham, C. B. Ramsey, I. Karavanic, F. H. Smith, E. Trinkaus, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 553 (2006).
38. M. Belmaker, E. Hovers, *Quat. Sci. Rev.* **30**, 3196 (2011).
39. P. Semal et al., *Am. J. Phys. Anthropol.* **138**, 421 (2009).
40. W. E. Banks et al., *PLoS ONE* **3**, e3972 (2008).
41. C. Stringer, in *Neanderthals Revisited: New Approaches and Perspectives*, K. Harvati, T. Harrison, Eds. (Springer, New York, 2006), pp. 315–323.
42. C. Finlayson, *Trends Ecol. Evol.* **20**, 457 (2005).
43. J. R. Stewart, *Quat. Int.* **137**, 35 (2005).
44. M. Bradtmöller, A. Pastoors, B. Weninger, G.-C. Weninger, *Quat. Int.* **247**, 38 (2012).
45. J. Krause et al., *Nature* **449**, 902 (2007).
46. A. Gibbons, *Science* **333**, 1084 (2011).
47. V. Fabre, S. Condemni, A. Degioanni, *PLoS ONE* **4**, e5151 (2009).
48. J. Krause et al., *Nature* **464**, 894 (2010).
49. D. Reich et al., *Nature* **468**, 1053 (2010).
50. L. Orlando et al., *Curr. Biol.* **16**, R400 (2006).
51. C. Stringer, *Philos. Trans. R. Soc. London Ser. B* **357**, 563 (2002).
52. J. S. Carrión, J. Rose, C. Stringer, *Quat. Sci. Rev.* **30**, 1281 (2011).
53. E. Indriati et al., *PLoS ONE* **6**, e21562 (2011).
54. P. Brown et al., *Nature* **431**, 1055 (2004).
55. M. J. Morwood, W. L. Jungers, *J. Hum. Evol.* **57**, 640 (2009).
56. R. Grün et al., *J. Hum. Evol.* **49**, 316 (2005).
57. C. Stringer, *The Origin of Our Species* (Allen Lane, London, 2011).
58. P. Endicott, S. Y. Ho, C. Stringer, *J. Hum. Evol.* **59**, 87 (2010).
59. E. Trinkaus et al., *Proc. Natl. Acad. Sci. U.S.A.* **100**, 11231 (2003).
60. C. Gamble, W. Davies, P. Pettitt, M. Richards, *Philos. Trans. R. Soc. London Ser. B* **359**, 243 (2004).
61. T. Goebel, M. R. Waters, D. H. O'Rourke, *Science* **319**, 1497 (2008).
62. C. P. E. Zollikofer, M. S. Ponce de León, *Semin. Cell Dev. Biol.* **21**, 441 (2010).
63. R. Dennell, *Episodes* **31**, 207 (2008).
64. R. Potts, *Evol. Anthropol.* **7**, 81 (1998).
65. M. J. Walker, J. Ortega, K. Parmová, M. V. López, E. Trinkaus, *Proc. Natl. Acad. Sci. U.S.A.* **108**, 10087 (2011).
66. C. Stringer, *Homo britannicus: The Story of Life in Britain* (Allen Lane, London, 2006).
67. T. W. Holliday, *Am. J. Phys. Anthropol.* **104**, 245 (1997).
68. C. S. Coon, *The Origin of the Races* (Knopf, New York, 1962).
69. E. Trinkaus, in *Aspects of Human Evolution*, C. B. Stringer, Ed. (Taylor & Francis, London, 1981), pp. 187–224.
70. T. C. Rae, T. Koppe, C. B. Stringer, *J. Hum. Evol.* **60**, 234 (2011).
71. T. W. Holliday, *J. Hum. Evol.* **32**, 423 (1997).
72. T. D. Weaver, C. C. Roseman, C. B. Stringer, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 4645 (2008).
73. P. Endicott, S. Y. Ho, M. Metspalu, C. Stringer, *Trends Ecol. Evol.* **24**, 515 (2009).
74. R. E. Green et al., *Science* **328**, 710 (2010).
75. J. Mallet, *Trends Ecol. Evol.* **20**, 229 (2005).
76. M. L. Arnold, *Natural Hybridization and Evolution* (Oxford Univ. Press, Oxford, 1997).
77. C. J. Jolly, *Am. J. Phys. Anthropol.* **116** (suppl. 33), 177 (2001).
78. D. Zinner, M. L. Arnold, C. Roos, *Evol. Anthropol.* **20**, 96 (2011).
79. G. M. Hewitt, *Mol. Ecol.* **10**, 537 (2001).
80. P. R. Grant, B. R. Grant, *Am. Nat.* **149**, 1 (1997).
81. M. Currat, L. Excoffier, *Proc. Natl. Acad. Sci. U.S.A.* **108**, 15129 (2011).
82. D. Reich et al., *Am. J. Hum. Genet.* **89**, 516 (2011).
83. M. Rasmussen et al., *Science* **334**, 94 (2011).
84. M. I. Bird, A. Taylor, C. Hunt, *Quat. Sci. Rev.* **24**, 2228 (2005).
85. C. I. Smith, A. T. Chamberlain, M. S. Riley, C. Stringer, M. J. Collins, *J. Hum. Evol.* **45**, 203 (2003).
86. M. F. Hammer, A. E. Woerner, F. L. Mendez, J. C. Watkins, J. D. Wall, *Proc. Natl. Acad. Sci. U.S.A.* **108**, 15123 (2011).
87. I. Crevecoeur et al., *Am. J. Phys. Anthropol.* **550**, 87 (2010).
88. K. Harvati et al., *PLoS ONE* **6**, e24024 (2011).
89. S. Ghirotto, F. Tassi, A. Benazzo, G. Barbujani, *Am. J. Phys. Anthropol.* **146**, 242 (2011).
90. L. Abi-Rached et al., *Science* **334**, 89 (2011).
91. L. Parducci et al., *Science* **335**, 1083 (2012).
92. J. Imbrie et al., in *Milankovitch and Climate*, A. L. Berger et al., Eds. (Reidel, Dordrecht, Netherlands, 1984), pp. 269–305.
93. D. A. Meese et al., *J. Geophys. Res.* **102**, 26411 (1997).
94. J. Pross et al., *Sci. Drill.* **5**, 30 (2007).
95. Y. J. Wang et al., *Science* **294**, 2345 (2001).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/335/6074/1317/DC1
Table S1
References (96–128)

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The Fern Sporangium: A Unique Catapult

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Spore dispersal in plants and fungi plays a critical role in the survival of species and is thus under strong selective pressure. As a result, various plant and fungal groups have evolved ingenious mechanisms to disperse their spores effectively (1, 2). Many of these mechanisms use the same physical principles as man-made devices but often achieve better performance. One such dispersal mechanism is the cavitation-triggered catapult of fern sporangia. The sporangia open when dehydrating and use the stored elastic energy to power a fast closure motion that ultimately ejects the spores. The beauty of this dispersal mechanism and its similarity with medieval catapults have not escaped notice (7). All man-made catapults are equipped with a crossbar to stop the motion of the arm midway. Without it, catapults would launch their projectiles into the ground. This crossbar is conspicuously missing from the sporangium, suggesting that it should simply speed up to its closed conformation without ejecting the spores. We show that much of the sophistication of this ejection mechanism and the basis for its efficiency lie in the two very different time scales

associated with the sporangium closure. The simple structure of the sporangium belies the complexity of its action (Fig. 1A). Central to the ejection process is the annulus: a row of 12 to 13 cells that forms a crest to one side of a spherical capsule enclosing the spores. As the annulus cells lose water by evaporation, water tension builds up within them, thus forcing the thickened radial walls closer together and causing lateral walls to collapse internally (3, 4) (Fig. 1B and movie S1). The whole annulus is thus bent out of shape, much like an accordion in the hands of a musician. The strong change in curvature (Fig. 1, B and D) forces the opening of the sporangium at the stoma, thus exposing the spores. When water tension in the annulus cells reaches a critical value [about -9 MPa (5)], cavitation occurs within adjacent cells (6) (Fig. 1C, fig. S2, and movies S2 and S3). The annulus then closes by 30 to 40% within about $10 \mu\text{s}$, leading to a quick release of the energy stored in the annulus and expulsion of the spores at an initial velocity of up to 10 m s^{-1} (7). This corresponds to an acceleration of about $10^5 g$. This first phase is followed by a comparatively

slow relaxation to an 85% closed configuration in a few hundreds of ms. We interpret the two time scales as a fast inertial recoil of the annulus followed by a slow poroelastic dissipation (8) of the energy remaining in the annulus. The annulus walls are constituted of a tight network of cellulose fibers surrounded by water that flows to conform to their relative displacements. The tiny size of the pores (9) and thick walls (10) induce strong viscous losses (from Darcy's law) that dramatically slow down the annulus motion. This dynamic can be described by using a generalized viscoelastic Maxwell model that fits our data very well and integrates all the physical forces at play (Fig. 1E and fig. S3). The measured and predicted time scales are in good agreement both for the inertial (respectively 25 and $27 \mu\text{s}$) and the poroelastic (respectively 5.8 and 3 ms) regimes. The coexistence of these two widely different time scales allows the sporangium to release its spores efficiently without the use of structural elements to arrest the recoil motion.

A dozen cells placed in a row can fulfill all the functions of a medieval catapult, including the motive force for charging the catapult (water cohesion), energy storage (annulus wall), triggering mechanism (cavitation), and returning motion arrest (poroelastic behavior of the annulus wall).

References and Notes

1. C. T. Ingold, *Spore Liberation* (Clarendon, Oxford, 1965).
2. X. Noblin, S. Yang, J. Dumais, *J. Exp. Biol.* **212**, 2835 (2009).
3. O. Renner, *Jahrb. Wiss. Bot.* **56**, 647 (1915).
4. A. Ursprung, *Ber. Dtsch. Bot. Ges.* **33**, 153 (1915).
5. X. Noblin *et al.*, in *Proceedings of the 6th Plant Biomechanics Conference*, Cayenne, French Guyana, 16 to 21 November 2009.
6. K. T. Ritman, J. A. Milburn, *J. Exp. Bot.* **41**, 1157 (1990).
7. A. L. King, *Proc. Natl. Acad. Sci. U.S.A.* **30**, 155 (1944).
8. J. M. Skotheim, L. Mahadevan, *Proc. R. Soc. London A* **460**, 1995 (2004).
9. N. Carpita, D. Sabulase, D. Montezinos, D. P. Delmer, *Science* **205**, 1144 (1979).
10. K. Haider, *Planta* **44**, 370 (1954).

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Materials and Methods

Fig. S1 to S4

References

Movies S1 to S4

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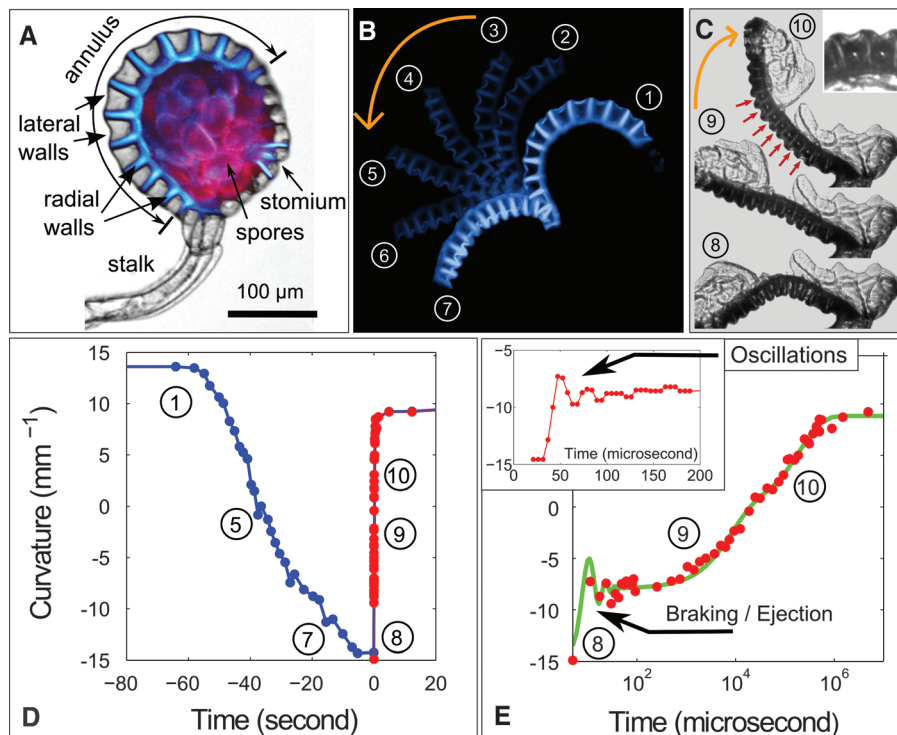


Fig. 1. (A) The sporangium of *Polypodium aureum*. Annulus geometry during sporangium opening in (B) and in (C) just before cavitation and 0.4 and 40 ms after cavitation. Note the seven cells that have cavitated (red arrows). (D) Annulus curvature during opening (blue) and closing (red) phases. (E) The closing phase in log-linear scale reveals the poroelastic relaxation; the green line represents the fit from our model. The numbers correspond to frames in (B) and (C). (Inset) Expanded view of the inertial oscillations.

Ultrastrong Coupling of the Cyclotron Transition of a 2D Electron Gas to a THz Metamaterial

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Artificial cavity photon resonators with ultrastrong light-matter interactions are attracting interest both in semiconductor and superconducting systems because of the possibility of manipulating the cavity quantum electrodynamic ground state with controllable physical properties. We report here experiments showing ultrastrong light-matter coupling in a terahertz (THz) metamaterial where the cyclotron transition of a high-mobility two-dimensional electron gas (2DEG) is coupled to the photonic modes of an array of electronic split-ring resonators. We observe a normalized coupling ratio, $\frac{\Omega}{\omega_c} = 0.58$, between the vacuum Rabi frequency, Ω , and the cyclotron frequency, ω_c . Our system appears to be scalable in frequency and could be brought to the microwave spectral range with the potential of strongly controlling the magnetotransport properties of a high-mobility 2DEG.

Enhancement and tunability of light-matter interaction is crucial for fundamental studies of cavity quantum electrodynamics (QED) and for applications in classical and quantum devices (1–4). The coupling between one cavity photon and one elementary electronic excitation is quantified by the vacuum Rabi frequency, Ω . The nonperturbative strong light-matter coupling regime is achieved when Ω is larger

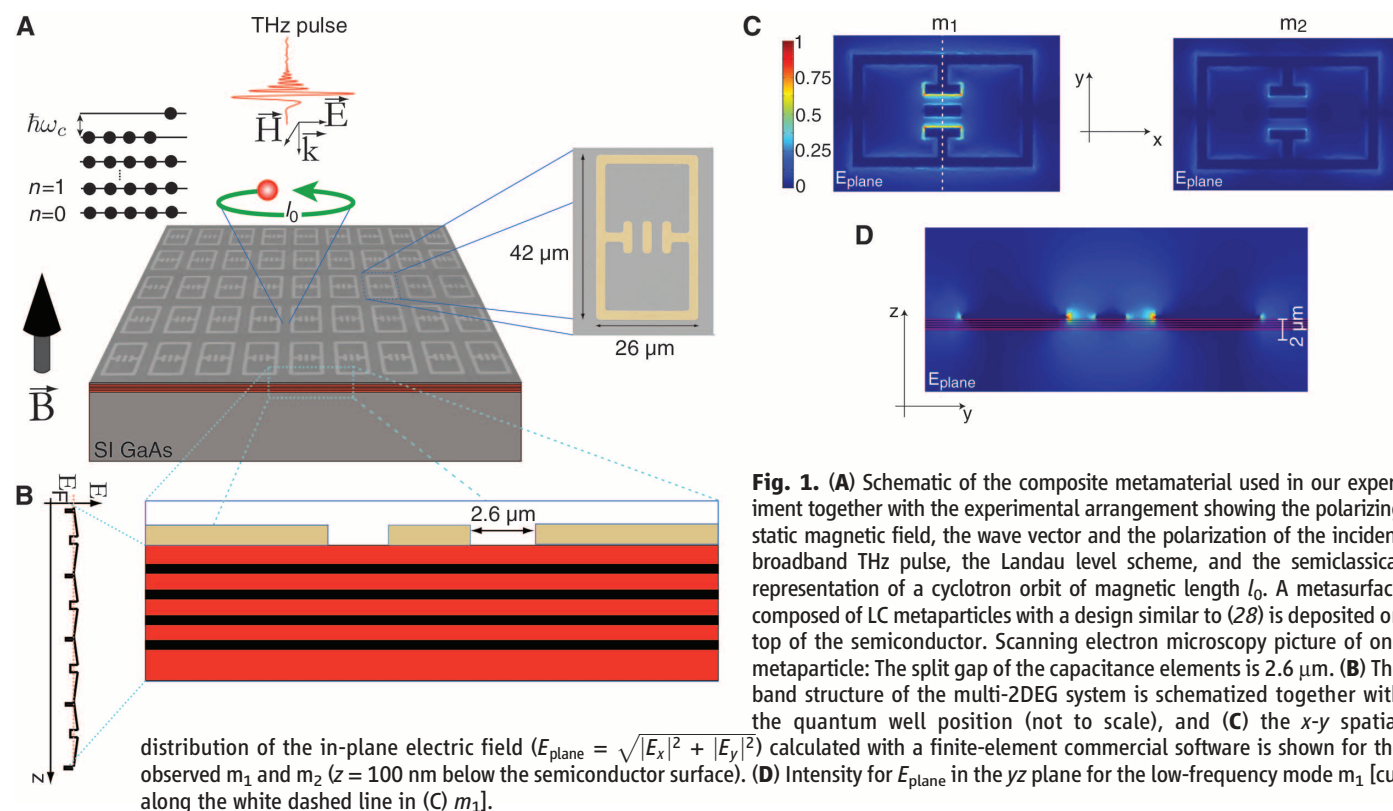
than the loss rates of the photons and electronic excitations. Recently, growing interest has been generated by the ultrastrong coupling regime (5–12), which is obtained when the vacuum Rabi frequency becomes an appreciable fraction of the unperturbed frequency of the system, ω . In such a regime, it is possible to modify the ground- and excited-state properties obtaining nonadiabatic cavity QED effects (5). Ex-

perimental progress has been achieved in two different solid-state systems: (i) microcavities embedding doped quantum wells (13–17), where the active electronic transition is between quantized subbands in the well, and (ii) superconducting quantum circuits in transmission line resonators (18, 19), where the photon field is coupled to artificial two-level atoms obtained with Josephson junctions.

We present experimental results on a high-mobility two-dimensional electron gas (2DEG) coupled to terahertz (THz) metamaterial resonators. The photon mode is coupled to the magnetic cyclotron transition of the 2DEG, obtained by applying a magnetic field perpendicular to the plane of the quantum wells (Fig. 1A). The cyclotron frequency is expressed by $\omega_c = \frac{eB}{m^*}$, where B is the applied magnetic field, e is the elementary charge, and m^* represents the electron effective mass. This highly controllable system is ideal for the study of strong coupling because the material excitation can be continuously tuned by changing the value of the applied magnetic field. The key physical aspect to

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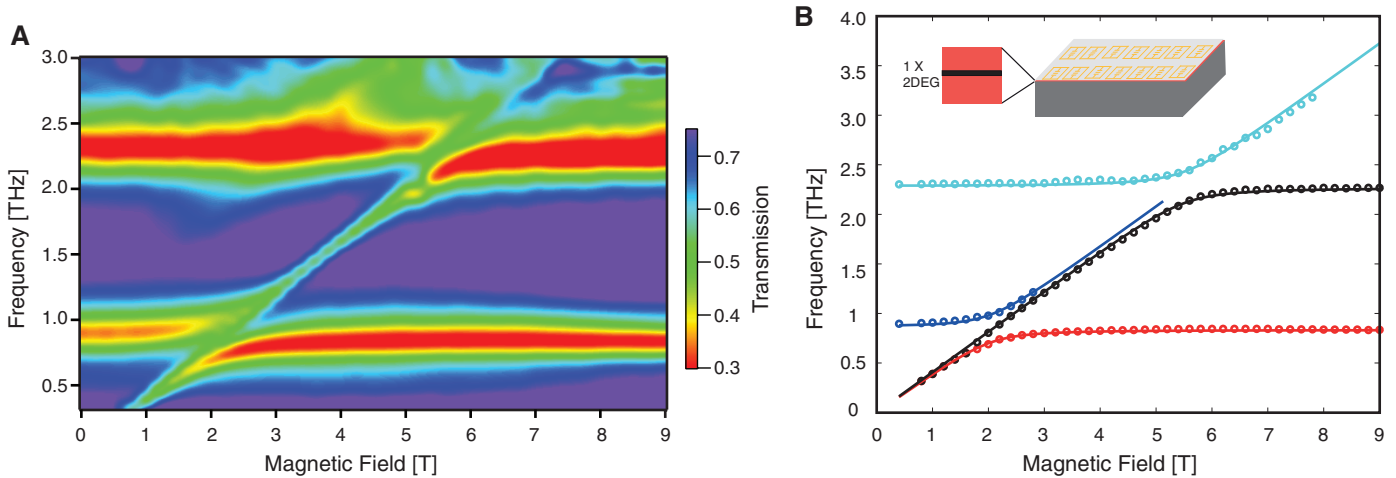


Fig. 2. (A) Transmission $|t|$ of the sample ($n_{\text{QW}} = 1$) as a function of B . The reference is a plain 2DEG sample without resonators on top, and the measurement is performed at temperature (T) = 2.2 K. (B) Best fit with the extracted transmitted minima positions for the two different transverse modes of the electronic split-ring resonator; the fitting parameter is $\frac{\Omega}{\omega}$.

highlight is the dependence of the optical dipole moment, d , for a cyclotron transition on the cyclotron orbit length. The dipole d scales as $d \sim e l_0 \sqrt{\nu}$ (20), where $l_0 = \sqrt{\hbar/eB}$ is the magnetic length and $\nu = \rho_{2\text{DEG}} 2\pi l_0^2$ is the filling factor of the 2DEG, with $\rho_{2\text{DEG}}$ being the electron areal density. This proportionality of the dipole with respect to l_0 allows to have gigantic dipole moments as soon as the cyclotron transition can be resolved. According to theoretical calculations valid for integer filling factors and for an optimized resonator geometry, the coupling ratio is expected to scale as $\frac{\Omega}{\omega_c} \sim \sqrt{\alpha n_{\text{QW}} \nu}$, where α is the fine structure constant and n_{QW} is the number of 2DEGs (20). For high filling factors, this coupling ratio is predicted to assume values even larger than unity (corresponding to transitions in the microwave range). We used high-mobility 2DEG based on GaAs material system (21), and we realized our experiments in the THz region of the electromagnetic spectrum. These frequencies, for our material system, correspond to magnetic fields of the order of a few tesla, and optical experiments were conducted by using broadband THz pulses generated with ultrafast lasers (22). A THz-time domain spectroscopy (TDS) system (bandwidth 0.1 to 3 THz) (23) is coupled to a split-coil superconducting magnet to probe sample transmission (24).

Our THz metamaterial integrates the 2DEG with a metasurface of electronic split-ring resonators (Fig. 1A). These resonators (25–28) exhibit electric field enhancement over strongly subwavelength volumes (29), making them ideal candidates to reach extreme couplings in the mid-infrared and THz range, where long wavelength radiation has to interact with quantum well systems typically extending over lengths of some micrometers (30). Moreover, the enhanced in-plane (x - y) electric field couples efficiently to the cyclotron transition when the magnetic field is applied perpendicularly to the plane of the layers and parallel to the wave vector of the incident

THz pulse (Fig. 1A). Resonators were deposited on top of the 2DEG by conventional photolithography, metallization with Ti/Au (5/250 nm), and lift-off technique.

At zero magnetic field, we observe two resonances, m_1 and m_2 , whose origin is qualitatively different: the lowest frequency mode ($f_1 \approx 0.9$ THz) is attributed to the LC resonance, where counterpropagating currents circulate in the inductive part and the electric field is enhanced mainly in the capacitor gap (26) (Fig. 1C). The second mode ($f_2 \approx 2.3$ THz) is attributed to the “cut wire” behavior, where a $\lambda/2$ kind of resonance is excited along the sides of the metaparticle (27). These values correspond well to simulations with 3D FE modeling (text S1). The two modes of the split-ring resonator also have different transverse wave vectors, as is evident looking at the different field distributions (Fig. 1C). The presence of conductive layers underneath alters the frequency and the quality factor of these resonances.

We observe a value of $Q_{1\text{THz}}^{(\text{ins})} \approx 4.3$ for an insulating substrate and $Q_{1\text{THz}}^{(2\text{DEG})} \approx 3.1$ when the resonator is deposited on top of the single 2DEG sample. The values for the second resonance results are less affected, yielding $Q_{2.3\text{THz}}^{(\text{ins})} \approx 5.3$ and $Q_{2.3\text{THz}}^{(2\text{DEG})} \approx 5.3$ (a more detailed analysis can be found in text S1). In contrast to atomic systems, we can realize strong light-matter coupling physics with resonators displaying extremely low quality factors. The giant value of the coupling constant $\Omega \sim \sqrt{n_{\text{QW}} \rho_{2\text{DEG}}}$ typical of intersubband systems (5) together with the high electric field enhancement of subwavelength metallic resonators ($V_{\text{cav}} \approx 8 \times 10^{-17} \text{ m}^3$ in our case) allow the observation of cavity polaritons in a system where both components are in principle highly dissipative.

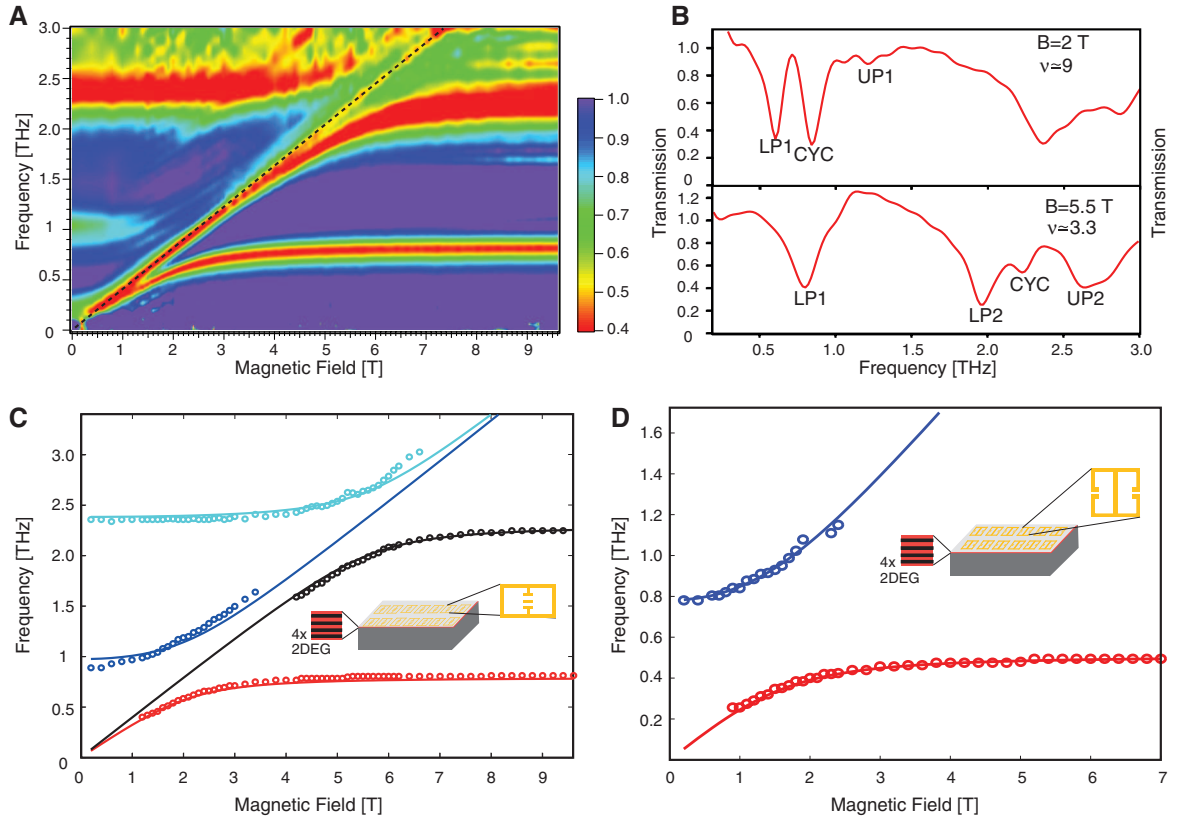
In the data reported in Fig. 2A, we observe the evolution of the sample transmission $|t| = \left| \frac{E_{\text{Mm}}(B)}{E_{2\text{DEG}}(0)} \right|$

as a function of the applied magnetic field (normalized to the electric field $E_{2\text{DEG}}(0)$ of the reference 2DEG wafer at $B = 0$ T). One 2DEG ($n_{\text{QW}} = 1$ and electron density $\rho_{2\text{DEG}}^{(1)} = 3.2 \times 10^{11} \text{ cm}^{-2}$) is used as an active medium and placed 100 nm below the surface: Its cyclotron resonance can couple to the resonator modes. As the magnetic field is swept, a profound modification of the sample transmission is observed. The possibility to tune in a continuous way the material excitation allows us to follow the evolution of polaritonic states as the system is driven from the uncoupled regime to the strongly coupled one (31). We observed two successive anticrossings when the cyclotron energy matches the first and the second resonator modes.

In Fig. 2B, we extracted the positions of the minima of sample transmission and plotted the dispersion curves for the polariton eigenvalues as a function of the magnetic field. The curves are calculated by using a full quantum mechanical treatment of the system, obtained generalizing the theory described in (20) to the case of a zero-dimensional resonator exhibiting two modes with different transverse wave vectors (24). Following a bosonization procedure, we have derived the different contributions to the total Hamiltonian and have diagonalized it by using the Hopfield-Bogoliubov method. The ideal resonator we have considered in the analytical treatment is different from the real splitting one. However, we emphasize that this would introduce only a form factor in the matrix element calculation.

In order to fit the experimental data, we need to know the resonator modes frequencies (f'_1 and f'_2) as well as the strength of their couplings (Ω_1 and Ω_2). For each cavity mode, we assumed that the asymptotic value of the corresponding lower polariton branch coincides with the frequency of the unloaded resonator ($f'_1 = 0.83$ THz and $f'_2 = 2.26$ THz) (20, 24). The coupling strength, $\frac{\Omega}{\omega}$, for the two modes

Fig. 3. (A) Transmission $|t|$ of the sample ($n_{\text{QW}} = 4$) as a function of B . The reference is a plain 2DEG sample without resonators on top, and the measurement is performed at $T = 10$ K. The black dotted line highlights the cyclotron signal coming from the uncoupled material that is left between the resonators. (B) Sections in the two anti-crossing regions for the sample transmission. (C) Best fit with the extracted transmitted minima positions for the two orthogonal modes of the split-ring electronic resonator; the fitting parameter is $\frac{\Omega_1}{\omega_1}$. (D) Best fit with the extracted transmitted minima positions for the $f = 500$ GHz resonator and $n_{\text{QW}} = 4$ measured at $T = 10$ K; the fitting parameter is $\frac{\Omega_2}{\omega_2}$. (Inset) Scheme of the 500-GHz resonator.



cannot be directly measured; we thus applied a best-fit procedure following the least square method, analogously to what done in (14) (more details in text S3). The minimal error (24) is obtained for $\frac{\Omega_1}{\omega_1} = 0.17$ and $\frac{\Omega_2}{\omega_2} = 0.075$ (where $\omega_1 = 2\pi f_1'$ and $\omega_2 = 2\pi f_2'$). As expected, the coupling strength scales monotonously with v : for the measured density $\rho_{\text{2DEG}}^{(1)}$ we have $v(B) = v(2T) \simeq 6.5$ for the first mode and $v(B) = v(5.5T) \simeq 2.4$ for the second one.

To increase the coupling strength, we kept the resonator geometry, and hence the frequency constant, and increased the effective number of carriers in the system. A new sample was prepared with $n_{\text{QW}} = 4$ wells and an electron density per well of $\rho_{\text{2DEG}}^{(4)} = 4.45 \times 10^{11} \text{ cm}^{-2}$ (Fig. 1B scheme and materials and methods). Sample transmission as a function of the applied magnetic field (Fig. 3A) shows that the system is driven deeply into the ultrastrong coupling regime. The polaritonic line widths display narrowing as the low quality cavity mode is mixed with the cyclotron resonance (Fig. 3B). Following the fitting procedure previously described, we observe a value of $\frac{\Omega_1}{\omega_1} = 0.36$ for the first resonance. Indeed, the effects of the antiresonant terms of the light-matter Hamiltonian start becoming relevant when the dimensionless ratio $\frac{\Omega}{\omega}$ is of the order of 0.1 (14). Because of the increased doping, the filling factor in the region of the anticrossing is $v \simeq 9$. As expected, the coupling ratio scales with $\sqrt{\rho_{\text{2DEG}} n_{\text{QW}}}$, and for the two samples at the

same resonant frequency we obtain experimen-

$$\text{tally } \frac{\left(\frac{\Omega_1}{\omega_1}\right)_{n_{\text{QW}}=4}}{\left(\frac{\Omega_1}{\omega_1}\right)_{n_{\text{QW}}=1}} = \frac{0.36}{0.17} = 2.11 \text{ and theoretically}$$

$\sqrt{\frac{4\rho_{\text{2DEG}}^{(4)}}{\rho_{\text{2DEG}}^{(1)}}} = 2.35$. The small discrepancy can be attributed to the different coupling of the quantum wells, which do not experience the same electric field of the resonator (Fig. 1D). The generalized expression of the coupling ratio is calculated in the case when all the wells are coupled in the same way to the resonator's field (20).

By further scaling the resonator frequency down to $f \simeq 500$ GHz with a slightly modified geometry (Fig. 3D inset, text S2, and fig. S5) and by using the sample with $n_{\text{QW}} = 4$ quantum wells, we could probe the regime where the polariton splitting at the anticrossing $2\hbar\Omega$ is larger than the bare cavity photon energy. In Fig. 3D, we report the positions of the minima of the sample transmission for the case of $f = 500$ GHz resonator together with the fitted dispersion curves. We measure a normalized ratio $\frac{\Omega_1}{\omega_1} = 0.58$ for a filling factor of $v(1.2T) \simeq 15.2$, which corresponds to $2\hbar\Omega \simeq 1.2\omega_c$ (fig. S6).

The generalization of the theory developed in (20) accounts for the depolarization shift in presence of a magnetic field (magnetoplasmon) originating from the long-wavelength part of the Coulomb interaction. We found that the renormalization of the cyclotron transition frequency is too small to allow the experimental resolution of the magnetoplasmon branch (Figs. 2A and

3A) in our experimental parameter regime, including the small wave vector condition $q l_0 \ll 1$, which is always satisfied because we are dealing with optical wave vectors.

We have observed ultrastrong light-matter coupling in a composite THz metamaterial measuring a normalized coupling ratio $\frac{\Omega}{\omega_c} = 0.58$. The impact of our results has to be considered also in the perspective of a change in the DC transport properties of the 2DEG, in analogy with what already observed by direct irradiation at lower frequencies (31).

These results should lead to the scaling of the frequency to lower values and to an increase of effective density to further enhance the coupling strength.

References and Notes

1. J. Raimond, M. Brune, S. Haroche, *Rev. Mod. Phys.* **73**, 565 (2001).
2. A. Wallraff *et al.*, *Nature* **431**, 162 (2004).
3. S. Christopoulos *et al.*, *Phys. Rev. Lett.* **98**, 126405 (2007).
4. K. Hennessy *et al.*, *Nature* **445**, 896 (2007).
5. C. Ciuti, G. Bastard, I. Carusotto, *Phys. Rev. B* **72**, 115303 (2005).
6. C. Ciuti, I. Carusotto, *Phys. Rev. A* **74**, 033811 (2006).
7. S. De Liberato, C. Ciuti, I. Carusotto, *Phys. Rev. Lett.* **98**, 103602 (2007).
8. M. H. Devoret, S. M. Girvin, R. J. Schoelkopf, *Ann. Phys. (Leipzig)* **16**, 767 (2007).
9. J. Bourassa *et al.*, *Phys. Rev. A* **80**, 032109 (2009).
10. V. M. Muravev, I. V. Andreev, I. V. Kukushkin, S. Schmult, W. Dietsche, *Phys. Rev. B* **83**, 075309 (2011).
11. T. Schwartz, J. A. Hutchison, C. Genet, T. W. Ebbesen, *Phys. Rev. Lett.* **106**, 196405 (2011).
12. P. Nataf, C. Ciuti, *Phys. Rev. Lett.* **104**, 023601 (2010).
13. D. Dini, R. Köhler, A. Tredicucci, G. Biasiol, L. Sorba, *Phys. Rev. Lett.* **90**, 116401 (2003).

14. A. Anappara *et al.*, *Phys. Rev. B* **79**, 213303 (2009).
15. G. Günter *et al.*, *Nature* **458**, 178 (2009).
16. Y. Todorov *et al.*, *Phys. Rev. Lett.* **105**, 196402 (2010).
17. M. Geiser *et al.*, *Phys. Rev. Lett.* **108**, 106402 (2012).
18. T. Niemczyk *et al.*, *Nat. Phys.* **6**, 772 (2010).
19. P. Forn-Díaz *et al.*, *Phys. Rev. Lett.* **105**, 237001 (2010).
20. D. Hagenmüller, S. De Liberato, C. Ciuti, *Phys. Rev. B* **81**, 235303 (2010).
21. V. Umansky *et al.*, *J. Cryst. Growth* **311**, 1658 (2009).
22. P. Smith, D. Auston, M. Nuss, *IEEE J. Quantum Electron.* **24**, 255 (1988).
23. D. Grischkowsky, S. Keiding, M. van Exter, C. Fittinger, *J. Opt. Soc. Am. B* **7**, 2006 (1990).
24. Supporting material is available on Science Online.
25. D. Schurig *et al.*, *Science* **314**, 977 (2006); 10.1126/science.1133628.
26. H.-T. Chen *et al.*, *Nature* **444**, 597 (2006).
27. W. J. Padilla, A. J. Taylor, C. Highstrete, M. Lee, R. D. Averitt, *Phys. Rev. Lett.* **96**, 107401 (2006).
28. J. O'Hara, E. Smirnova, A. Azad, H. Chen, A. J. Taylor, *Active Passive Electron. Components* **2007**, 10.1155/2007/49691 (2007).
29. C. Walther, G. Scalari, M. I. Amanti, M. Beck, J. Faist, *Science* **327**, 1495 (2010).
30. D. Shelton *et al.*, *ACS Nano Lett.* **11**, 2104 (2011).
31. The magnetic tunability has been studied in the very different system of exciton-polaritons in the near-infrared; see J. Tignon *et al.*, *Phys. Rev. Lett.* **74**, 3967 (1995).
32. R. G. Mani *et al.*, *Nature* **420**, 646 (2002).

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Materials and Methods

SOM Text
Figs. S1 to S7

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Laser Scribing of High-Performance and Flexible Graphene-Based Electrochemical Capacitors

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Although electrochemical capacitors (ECs), also known as supercapacitors or ultracapacitors, charge and discharge faster than batteries, they are still limited by low energy densities and slow rate capabilities. We used a standard LightScribe DVD optical drive to do the direct laser reduction of graphite oxide films to graphene. The produced films are mechanically robust, show high electrical conductivity (1738 siemens per meter) and specific surface area (1520 square meters per gram), and can thus be used directly as EC electrodes without the need for binders or current collectors, as is the case for conventional ECs. Devices made with these electrodes exhibit ultrahigh energy density values in different electrolytes while maintaining the high power density and excellent cycle stability of ECs. Moreover, these ECs maintain excellent electrochemical attributes under high mechanical stress and thus hold promise for high-power, flexible electronics.

Batteries and electrochemical capacitors (ECs) stand at opposite ends of the spectrum in terms of their power and energy densities (*I*). Batteries store energy through electrochemical reactions and can exhibit high energy densities (on the order of 20 to 150 Wh/kg), whereas ECs, which store charge in electrochemical double layers (EDLs), can only achieve values of 4 to 5 Wh/kg (2–4). However, because ion flow is faster than redox reactions ECs can deliver much higher power densities. ECs are also generally maintenance free and display a longer shelf and cycle life, so they are often favored in many electronic applications (2–4).

An EC that combines the power performance of capacitors with the high energy density of batteries would represent a major advance in energy storage technology (5, 6), but this requires an electrode with higher and more accessible surface area than that of conventional EC electrodes while maintaining high conductivity. Graphene-based materials are attractive in this regard be-

cause of their mechanical and electrical properties as well as exceptionally high surface area. Recently, the intrinsic capacitance of single-layer graphene was reported to be $\sim 21 \mu\text{F}/\text{cm}^2$; this value now sets the upper limit for EDL capacitance for all carbon-based materials (7). Thus, ECs based on graphene materials could, in principle, achieve an EDL capacitance as high as $\sim 550 \text{ F/g}$ if their entire surface area could be used.

Currently, graphene-based materials derived from graphite oxide (GO) can be manufactured on the ton scale at low cost, making them potentially cost-effective materials for charge storage devices (8). Although these graphene-based materials have shown excellent power density and life-cycle stability, their specific capacitance (130 F/g in aqueous potassium hydroxide and 99 F/g in an organic electrolyte) still falls far below the theoretical value of 550 F/g calculated for single-layer graphene (9). A variety of other graphene-based materials derived from GO have also been used, yet the values of specific capacitance, energy density, and power density have remained lower than expected (10–13)—an effect often attributed to the restacking of graphene sheets during its processing as a result of the strong sheet-to-sheet van der Waals interactions. This reduction in the specific surface area of graphene accounts for the overall low capacitance. In addition, these ECs exhibited relatively low charge/discharge rates, which precludes their use for high-power applications. Recently, EC devices composed of curved graphene (14), activated graphene (15), and solvated graphene (16) have demonstrated enhanced performance in terms of energy density. However, further improvements in energy density are needed that do not sacrifice high power density. In particular, the

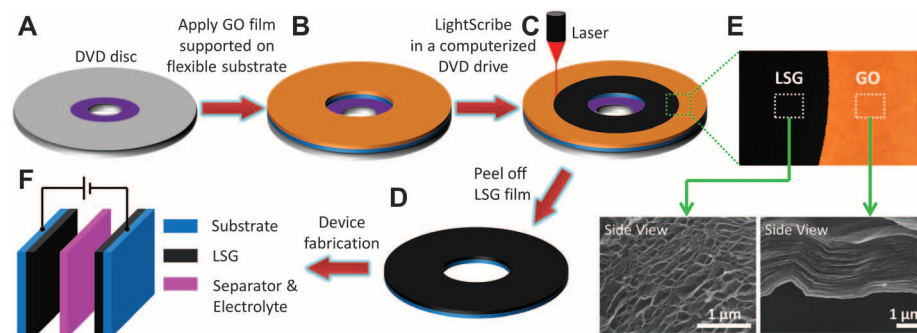


Fig. 1. Schematic illustration of the fabrication of laser-scribed graphene-based electrochemical capacitors. (A to D) A GO film supported on a flexible substrate is placed on top of a LightScribe-enabled DVD media disc, and a computer image is then laser-irradiated on the GO film in a computerized LightScribe DVD drive. (E) As shown in the photograph, the GO film changes from golden brown color to black as it is reduced to laser-scribed graphene. The low-power infrared laser changes the stacked GO sheets immediately into well-exfoliated few-layered LSG film, as shown in the cross-sectional SEM images. (F) A symmetric EC is constructed from two identical LSG electrodes, ion-porous separator, and electrolyte.

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production of mechanically robust graphene electrodes with large thickness ($\sim 10\ \mu\text{m}$ or higher) and high surface-to-volume ratio in a binderfree process would result in high power and high energy density ECs (5).

Here, we present a strategy for the production of graphene-based ECs through a simple all-solid-state approach that avoids the restacking of graphene sheets. The process is schematically illustrated in Fig. 1. Initially, a thin film of GO dispersed in water was drop-cast onto a flexible substrate (fig. S1). Irradiation of the film with an infrared laser inside an inexpensive commercially available LightScribe CD/DVD optical drive, as discussed in (17), reduces the GO to laser-scribed graphene (LSG), as indicated by the change in film color from golden brown to black. Analysis of cross sections of the film with scanning electron microscopy showed that the initially stacked GO sheets were converted into well-exfoliated LSG sheets through laser irradiation (Fig. 1E and fig. S2). The resulting LSG films showed excellent conductivity ($1738\ \text{S/m}$) as opposed to 10 to 100 S/m for activated carbons, the state-of-the-art material used in commercial devices (18). Additionally, LSG shows excellent mechanical flexibility with only $\sim 1\%$ change in the electrical resistance of the film after 1000 bending cycles [supporting online material (SOM) section 2 and fig. S3]. Thus, LSG can be directly used as EC

electrodes without the need for any additional binders or conductive additives. More importantly, these properties allow LSG to act as both the active material and current collector in the EC. The combination of both functions in a single layer leads to a simplified and lightweight architecture. Thus, a device can be readily made by sandwiching an ion porous separator [Celgard 3501 (Celgard, Charlotte, NC)] between two identical LSG electrodes. The devices are super-thin with a total thickness of $<100\ \mu\text{m}$, making them potentially useful in microdevice applications (fig. S4) (19). Other devices can be made by putting LSG on porous substrates such as a nitrocellulose membrane or photocopy paper or on conductive aluminum foil, which is often used in commercial devices (figs. S1 and S7). Therefore, LSG electrochemical capacitors (LSG-ECs) can be readily made into different designs, including stacked and spirally wound structures to target different applications.

The LSG electrodes we have fabricated satisfy the critical features for high-performance ECs. First, the large and accessible specific surface area of the LSG ($1520\ \text{m}^2/\text{g}$ compared with 1000 to 2000 m^2/g for a typical activated carbon material) results in a sizeable charge storage capacity and accounts for the high areal and volumetric stack capacitances observed. Second, the LightScribe laser causes the simultaneous re-

duction and exfoliation of GO sheets and produces an open network of LSG (Fig. 1). This structure prevents the agglomeration of graphene sheets, which has been a major barrier in achieving the full potential of graphene-based ECs. The network structure of LSG has open pores, which helps facilitate the electrolyte accessibility to the electrode surfaces. This offers an opportunity to optimize the ionic diffusion in LSG electrodes, which is crucial for charging the EDL, and generates high-power ECs. Last, LSG possesses excellent electronic conductivity, which is another key factor for achieving high power. These three properties taken together and optimized could, in theory, produce landmark performances in EC electrodes. Working with these properties, three-dimensional composite electrodes have been successfully used to make batteries with relatively high energy density and fast charge/discharge rates (20). Although activated carbons can provide high surface area, the difficulty of controlling their pore structure and pore size distribution has so far limited the energy densities and rate capabilities of commercial ECs (21).

In order to demonstrate the superior performance of LSG electrodes for electrochemical energy storage, we assembled symmetric LSG-ECs using polyethylene terephthalate (PET) as a thin flexible substrate and an aqueous electrolyte of 1.0 M H_3PO_4 . The EC performance was

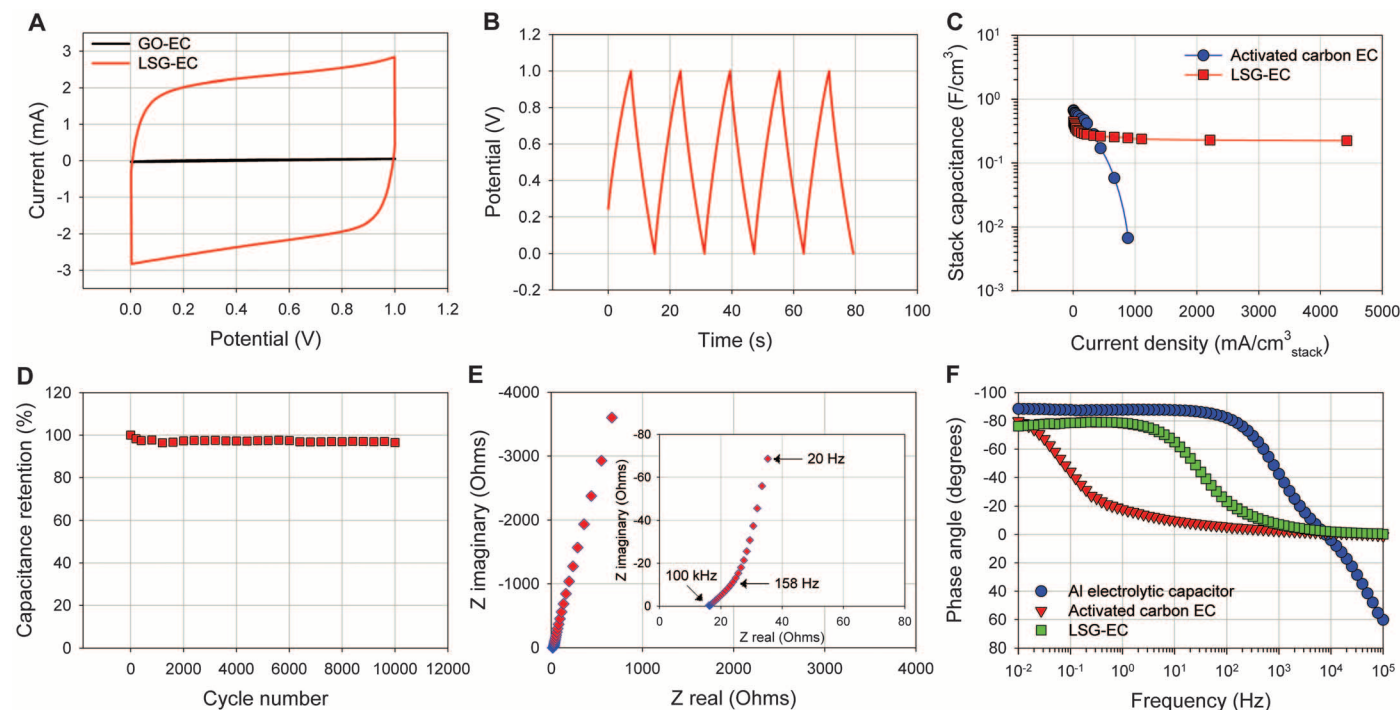


Fig. 2. Evaluation of the performance of an LSG electrochemical capacitor in aqueous 1.0 M H_3PO_4 solution. (A) Cyclic voltammetry of LSG- and GO-ECs at a scan rate of 1000 mV/s. A rectangular CV shape is observed for the LSG-EC, indicating an efficient double-layer formation. (B) Galvanostatic charge/discharge (CC) curves of an LSG-EC measured at a high current density of 10 A/g. (C) The volumetric stack capacitance of an LSG-EC is calculated from the CC curves at different charge/discharge current densities. Data obtained from a commercial activated carbon EC are shown for comparison.

As can be seen, an LSG-EC can sustain ultrahigh current density operation, indicating the potential for ultrahigh-power delivery. (D) The LSG-EC shows excellent cyclic stability and retains $>96.5\%$ of its initial response after 10,000 cycles. (E) Complex plane plot of the impedance of a LSG-EC, with a magnification for the high-frequency region in the inset. (F) Impedance phase angle versus frequency for a LSG-EC and a commercial activated carbon EC. The -45° phase angle occurs at $\sim 30\ \text{Hz}$ for the LSG-EC and at $\sim 0.1\ \text{Hz}$ with the commercial EC.

analyzed through both cyclic voltammetry (CV) and galvanostatic charge/discharge (CC) experiments, as shown in Fig. 2. In comparison with GO, the LSG-EC shows an enhanced electrochemical performance with a nearly rectangular CV shape at a scan rate of 1000 mV/s, which is indicative of nearly ideal capacitive behavior (Fig. 2A) even though no metal current collector, binders, or electroactive additives were used, as is the case in commercial ECs. Additionally, the LSG-EC is robust enough to be charged and discharged over a wide range of scan rates (100 to 10,000 mV/s) and still maintain its nearly ideal rectangular CV shape (figs. S5 to S7). Figure 2B shows the nearly triangular shape of the CC curves obtained at a high current density of 10 A/g of LSG per electrode (abbreviated 10 A/g_{LSG/electrode}). This is indicative of the formation of an efficient EDL and fast ion transport within the LSG electrodes. In addition, these CC curves show only a small voltage drop of 0.018 V at the start of the discharge curve, indicating a device with a low equivalent series resistance (ESR). We measured the specific capacitance from CC curves over a wide range of charge/discharge current densities. Here, the areal and volumetric capacitance of the stack (this includes the flexible substrate,

the current collector, the active material, and the separator) were calculated and compared with a commercial activated-carbon EC (AC-EC) tested under the same dynamic conditions. Although the AC-EC shows a slightly higher volumetric capacitance at low charge/discharge rates, its capacitance falls off quickly at higher rates, whereas the LSG-EC continues to provide high capacitance even when operated at very high rates (Fig. 2C and fig. S8). In addition, the areal capacitance of the LSG-EC was calculated to be 3.67 mF/cm² [and 4.04 mF/cm² in 1.0 M H₂SO₄ (figs. S5 to S7)] at 1 A/g_{LSG/electrode}. The device also shows a very high rate capability while still maintaining a capacitance of more than 1.84 mF/cm², even when the EC is operated at an ultrafast charge/discharge rate of 1000 A/g_{LSG/electrode}. This is comparable with values reported in the literature for micro-devices and thin-film ECs at much lower current charge/discharge rates (0.4 to 2 mF/cm²) (5, 13). As is shown in fig. S6, these ECs can be efficiently charged/discharged on the 0.1-s time scale. Additionally, the LSG-EC retained 96.5% of its initial capacitance after 10,000 cycles (Fig. 2D).

Electrochemical impedance spectroscopy (EIS) confirmed the fast ion transport within the LSG electrodes. A complex plan plot of the impedance

data of the LSG-EC is shown in Fig. 2E with an expanded view provided in the inset. The device displays a pure capacitive behavior, even at high frequencies of up to ~158 Hz. The series resistance of the device is estimated to be ~16 ohms. This value can be attributed to the contact resistance of the device with the external circuit that could be reduced by using current collectors. The dependence of the phase angle on the frequency for the LSG-EC, AC-EC, and an aluminum electrolytic capacitor is shown in Fig. 2F. For frequencies up to 10 Hz, the phase angle of the LSG-EC is close to -90°, which suggests that the device functionality is close to that of an ideal capacitor. The characteristic frequency f_0 for a phase angle of -45° is 30 Hz for the LSG-EC. This frequency marks the point at which the resistive and capacitive impedances are equal (22). The corresponding time constant τ_0 ($=1/f_0$) equals 33 ms compared with 10 s for the conventional AC-EC and 1 ms for the aluminum electrolytic capacitor. This rapid frequency response of the LSG-EC can be accounted for by the large and accessible surface area of the LSG, whose exposed flat sheets enhance the ion transport rate in the device (23). This is consistent with results reported recently for an EC made from vertically

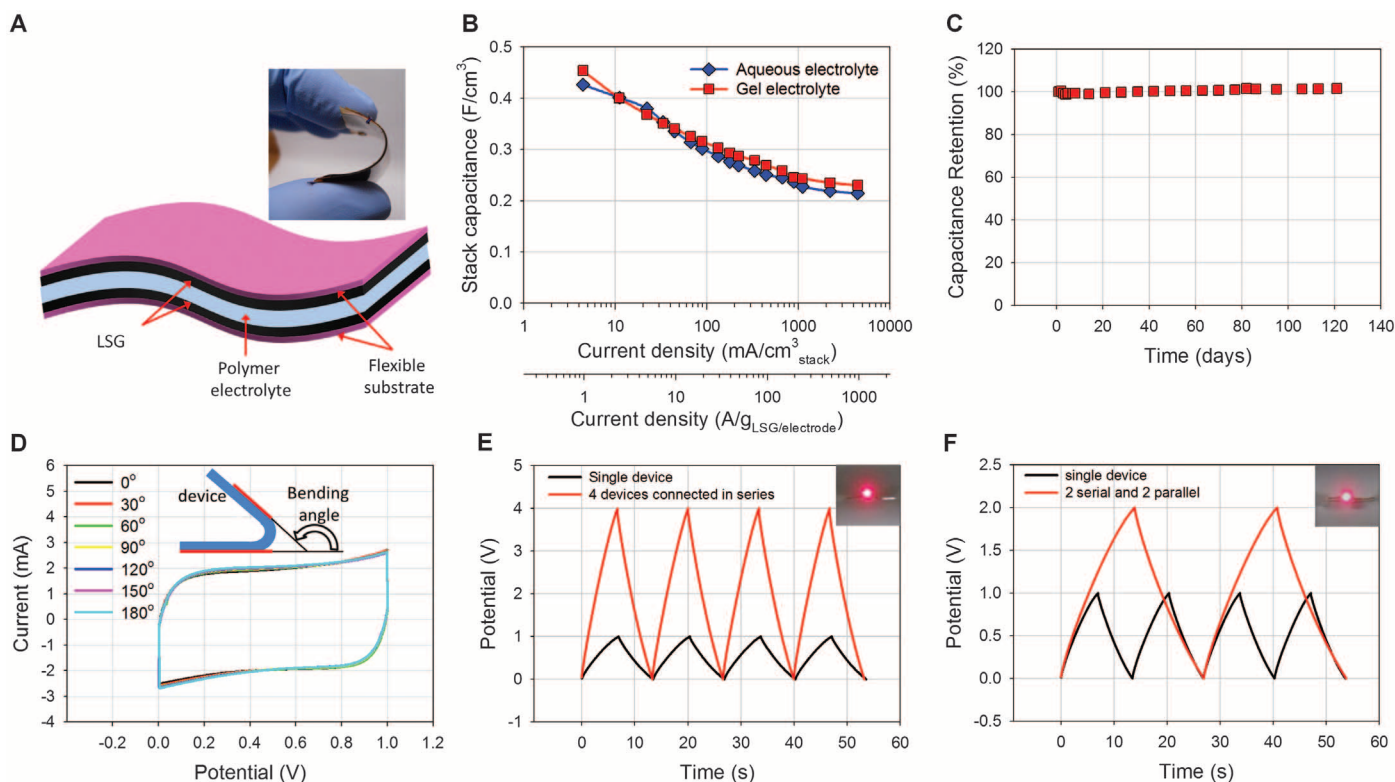


Fig. 3. Design and fabrication of a flexible, all-solid-state LSG electrochemical capacitor. (A) A schematic diagram of the all-solid-state LSG-EC illustrates that the gelled electrolyte can serve as both the electrolyte and separator. (Inset) A digital photograph showing the flexibility of the device. (B) A comparison between performances of LSG-EC using gelled versus aqueous electrolytes. Both devices show similar capacitance values at all the tested charge/discharge current densities. (C) A shelf-life test shows excellent stability for over 4 months without any obvious degradation. (D) Bending the device

has almost no effect on its performance, as seen in these CVs collected at a scan rate of 1000 mV/s. Galvanostatic charge/discharge curves for four tandem ECs connected (E) in series, and (F) in a combination of series and parallel. A single device is shown for comparison. Both the tandem devices and the single device were operated at the same constant current charge/discharge. The serial connection extends the output voltage to 4 V (versus 1 V for a single device), whereas the output voltage and current can both be doubled with the serial-parallel connection. (Insets) The glow of an LED when powered by tandem LSG-ECs.

oriented graphene nanosheets grown directly on metal current collectors (24–26) and carbon nanotube electrodes made with an electrophoretic deposition technique (27, 28).

The future development of multifunctional flexible electronics such as roll-up displays, photovoltaic cells, and even wearable devices presents new challenges for designing and fabricating lightweight, flexible energy storage devices (29). Commercially available ECs consist of a separator sandwiched between two electrodes with liquid electrolyte, which is then either spirally wound and packaged into a cylindrical container or stacked into a button cell (3). Unfortunately, these device architectures not only suffer from the possible harmful leakage of electrolytes, but their design makes it difficult to use them for practical flexible electronics. We replaced the liquid electrolyte with poly(vinyl alcohol) (PVA)- H_3PO_4 polymer gelled electrolyte, which also acts as the separator (Fig. 3A, device structure). This electrolyte reduced the device thickness and weight compared with phosphoric acid and simplified the fabrication process because it does not require any special packaging materials. As demonstrated in Fig. 3B, at any given charge/discharge rate the specific capacitance values for the all-solid-state device were comparable with those obtained with

an aqueous electrolyte. The high-rate performance of this device can be accounted for by the porous structure of the LSG electrodes, which can effectively absorb the gelled electrolyte and act as an electrolyte reservoir to facilitate ion transport and minimize the diffusion distance to the interior surfaces (30). Another key factor is that LSG electrodes are binderfree—thus, enabling a reduction in interfacial resistance and enhancing the electrochemical reaction rate. The device performance was completely stable over 4 months of testing (Fig. 3C). As with the aqueous LSG-EC, the flexible all-solid-state LSG-EC maintains its excellent cycling stability: >97% of the initial capacitance was maintained even after 10,000 cycles (fig. S10).

In order to evaluate under real conditions the potential of this all-solid-state LSG-EC for flexible energy storage, a device was placed under constant mechanical stress and its performance analyzed. The CV performance of this device when tested under different bending conditions is shown in Fig. 3D. The bending had almost no effect on the capacitive behavior; it can be bent arbitrarily without degrading performance. Moreover, the stability of the device was tested for more than 1000 cycles while in the bent state, with only ~5% change in the device capacitance (fig. S11). This performance durability can be

attributed to the high mechanical flexibility of the electrodes along with the interpenetrating network structure between the LSG electrodes and the gelled electrolyte. The electrolyte solidifies during the device assembly and acts like a glue that holds all the device components together, improving the mechanical integrity and increasing its cycle life even when tested under extreme bending conditions. Because this remarkable performance has yet to be realized in commercial devices, these ECs may be ideal for next-generation flexible, portable electronics.

Portable equipment often require cells packaged either in series, in parallel, or in combinations of the two in order to meet energy and power requirements. For example, laptop batteries commonly have four 3.6-V lithium ion cells connected in series to achieve a voltage of 14.4 V, and two in parallel to increase the capacity from 2400 mAh to 4800 mAh (31). Thus, it would be of interest to develop an EC that could exhibit control over the operating voltage and current by using tandem serial and parallel assemblies with minimal energy losses. The performances of a set of tandem LSG-EC were evaluated by assembling four devices both in series and in parallel configurations. Compared with a single EC, which operates at 1.0 V, the tandem series ECs exhibited a 4.0-V charge/discharge voltage window (Fig. 3E). In the parallel assembly, the output current increased by a factor of 4, and thus the discharge time was four times that of a single device when operated at the same current density (figs. S12 to S14). As expected, when the four ECs were combined two in series and two in parallel, both the output voltage and the runtime (capacitive current) increased by a factor of 2 under the same charge/discharge current (Fig. 3F). As with the single devices, the tandem devices show essentially perfect triangular CC curves with a miniscule voltage drop, which again indicates excellent capacitive properties with minimal internal resistance. Thus, when used in tandem, the LSG-ECs undergo minimal energy losses. As a demonstration, a tandem EC's ability to light a red light-emitting diode (LED) that operates at a minimum voltage of 2 V is shown in the Fig. 3, E and F, insets.

We also examined an organic electrolyte, which would allow the operation of the devices at higher voltages, thus achieving higher energy densities. In this case, tetraethylammonium tetrafluoroborate dissolved in acetonitrile was used because this is the most common organic electrolyte used in commercial devices (3). As shown in Fig. 4, the LSG-EC again exhibits enhanced performance and rate capabilities when compared with the commercial AC-EC; this is consistent with the data acquired in the aqueous and gelled electrolytes. Furthermore, the LSG-EC can be operated over a wider voltage window of 3 V. This EC offers a specific capacitance of up to 4.82 mF/cm^2 ($265 \text{ F/g}_{\text{LSG/electrode}}$) and retains a capacitance of 2.07 mF/cm^2 when operated at the ultrahigh current density of $1000 \text{ A/g}_{\text{LSG/electrode}}$ (fig. S15).

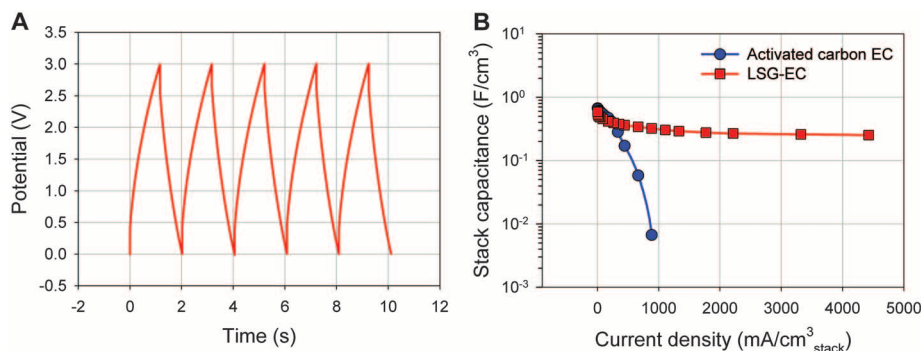
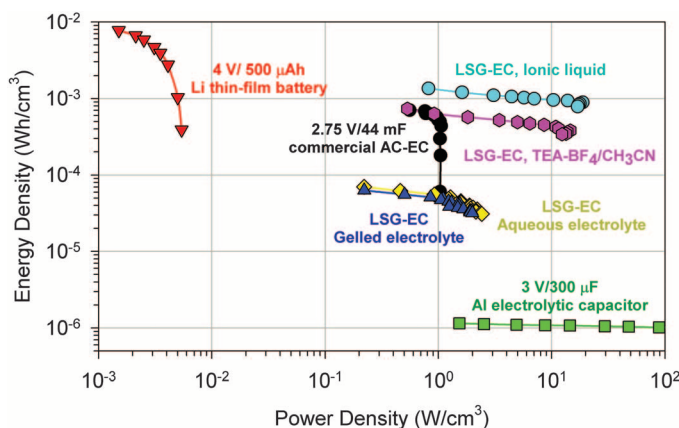


Fig. 4. The performance of a LSG electrochemical capacitor using an organic electrolyte of 1.0 M tetraethylammonium tetrafluoroborate dissolved in acetonitrile. (A) Galvanostatic charge/discharge curves of the device when operated at an ultrahigh current density of $250 \text{ A/g}_{\text{LSG/electrode}}$, showing near symmetric triangular shapes. (B) Stack capacitance values calculated from galvanostatic curves as a function of the applied charge/discharge current density showing the high rate performance possible with a LSG-EC versus that of an activated carbon EC.

Fig. 5. Energy and power densities of LSG-ECs compared with commercially available AC-EC, aluminum electrolytic capacitors, and a lithium thin-film battery. The LSG-ECs exhibit electrochemical energy storage with both ultrahigh power and energy densities. [The data for the Li thin-film battery are reproduced from (5) with permission from Nature Publishing Group]



Recently, room-temperature ionic liquids have been intensively studied as an attractive alternative to conventional electrolytes for ECs because of their high ion density, good thermal stability, and nonvolatility, as well as their wider potential window when compared with organic electrolytes (14, 32). We fabricated an LSG-EC using the ionic liquid 1-ethyl-3-methylimidazolium tetrafluoroborate (EMIMBF₄) that exhibited a specific capacitance as high as 5.02 mF/cm² (276 F/g_{LSG/electrode}) and at a wider potential window of 4 V (fig. S16). A prototype LSG-EC was made and encapsulated in the EMIMBF₄ electrolyte, charged at a constant potential of 3.5 V, and used to light up a red LED for ~24 min (movie S1).

In order to demonstrate the overall performance of the LSG-ECs using various electrolytes, a Ragone plot is shown in Fig. 5 comparing the performance of LSG-ECs with different energy storage devices designed for high-power microelectronics. This includes a commercial 2.75 V/44 mF AC-EC and a 500-μAh thin-film lithium battery and a 3 V/300 μF aluminum electrolytic capacitor, all tested under the same dynamic conditions (SOM section 9). The plot shows the volumetric energy density and power density of the stack for all the devices tested. The LSG-EC can exhibit energy densities of up to 1.36 mWh/cm³, a value that is approximately two times higher than that of the AC-EC. Additionally, LSG-ECs can deliver a power density of ~20 W/cm³, which is 20 times higher than that of

the AC-EC and three-orders of magnitude higher than that of the 500-μAh thin-film lithium battery. Although the electrolytic capacitor delivers ultrahigh power, it has an energy density that is three orders of magnitude lower than the LSG-EC. Because of the simplicity of the device architecture and the availability of the graphite oxide precursor, which is already manufactured on the ton scale, these LSG-ECs hold promise for commercial applications.

References and Notes

- C. Liu, F. Li, L.-P. Ma, H.-M. Cheng, *Adv. Energy Mater.* **22**, E28 (2010).
- D. S. Su, R. Schlögl, *ChemSusChem* **3**, 136 (2010).
- P. Simon, Y. Gogotsi, *Nat. Mater.* **7**, 845 (2008).
- J. R. Miller, P. Simon, *Science* **321**, 651 (2008).
- D. Pech *et al.*, *Nat. Nanotechnol.* **5**, 651 (2010).
- J. Chmiola, C. Largeot, P. L. Taberna, P. Simon, Y. Gogotsi, *Science* **328**, 480 (2010).
- J. Xia, F. Chen, J. Li, N. Tao, *Nat. Nanotechnol.* **4**, 505 (2009).
- M. Segal, *Nat. Nanotechnol.* **4**, 612 (2009).
- M. D. Stoller, S. Park, Y. Zhu, J. An, R. S. Ruoff, *Nano Lett.* **8**, 3498 (2008).
- Y. Wang *et al.*, *J. Phys. Chem. C* **113**, 13103 (2009).
- Z. Weng *et al.*, *Adv. Energy Mater.* **1**, 917 (2011).
- Y. Zhu *et al.*, *ACS Nano* **4**, 1227 (2010).
- W. Gao *et al.*, *Nat. Nanotechnol.* **6**, 496 (2011).
- C. G. Liu, Z. Yu, D. Neff, A. Zhamu, B. Z. Jang, *Nano Lett.* **10**, 4863 (2010).
- Y. Zhu *et al.*, *Science* **332**, 1537 (2011).
- X. Yang, J. Zhu, L. Qiu, D. Li, *Adv. Mater. (Deerfield Beach Fla.)* **23**, 2833 (2011).
- V. Strong *et al.*, *ACS Nano* 120215095449007 (2012).
- R. Chandrasekaran, Y. Soneda, J. Yamashita, M. Kodama, H. Hatori, *J. Solid State Electrochem.* **12**, 1349 (2008).

- S. W. Lee, B. M. Gallant, H. R. Byon, P. T. Hammond, Y. Shao-Horn, *Energy Environ. Sci.* **4**, 1972 (2011).
- H. Zhang, X. Yu, P. V. Braun, *Nat. Nanotechnol.* **6**, 277 (2011).
- L. L. Zhang, X. S. Zhao, *Chem. Soc. Rev.* **38**, 2520 (2009).
- P. L. Taberna, P. Simon, J. F. Fauvarque, *J. Electrochem. Soc.* **150**, A292 (2003).
- Y. Shim, Y. J. Jung, H. J. Kim, *J. Phys. Chem. C* **115**, 23574 (2011).
- X. Zhao *et al.*, *J. Power Sources* **194**, 1208 (2009).
- J. R. Miller, R. A. Outlaw, B. C. Holloway, *Science* **329**, 1637 (2010).
- J. R. Miller, R. A. Outlaw, B. C. Holloway, *Electrochim. Acta* **56**, 10443 (2011).
- C. Du, N. Pan, *J. Power Sources* **160**, 1487 (2006).
- C. Du, N. Pan, *Nanotechnology* **17**, 5314 (2006).
- H. Nishide, K. Oyaizu, *Science* **319**, 737 (2008).
- D. W. Wang, F. Li, M. Liu, G. Q. Lu, H.-M. Cheng, *Angew. Chem. Int. Ed.* **47**, 373 (2008).
- D. Andrea, *Battery Management Systems for Large Lithium Ion Battery Packs* (Artech House, Norwood, MA, 2010).
- J. F. Wishart, *Energy Environ. Sci.* **2**, 956 (2009).

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Materials and Methods
Figs. S1 to S16
References (33–39)
Movie S1

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Scaling Hetero-Epitaxy from Layers to Three-Dimensional Crystals

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Quantum structures made from epitaxial semiconductor layers have revolutionized our understanding of low-dimensional systems and are used for ultrafast transistors, semiconductor lasers, and detectors. Strain induced by different lattice parameters and thermal properties offers additional degrees of freedom for tailoring materials, but often at the expense of dislocation generation, wafer bowing, and cracks. We eliminated these drawbacks by fast, low-temperature epitaxial growth of Ge and SiGe crystals onto micrometer-scale tall pillars etched into Si(001) substrates. Faceted crystals were shown to be strain- and defect-free by x-ray diffraction, electron microscopy, and defect etching. They formed space-filling arrays up to tens of micrometers in height by a mechanism of self-limited lateral growth. The mechanism is explained by reduced surface diffusion and flux shielding by nearest-neighbor crystals.

Quantum structures based on epitaxially grown semiconductor layers are a playground for studying electron transport and optical properties, especially at low temperatures, where electron mobilities have reached values above 30 million cm²/V·s (1). The discovery of fundamental phenomena such as the fractional quantum Hall effect (2) can be traced to the perfection of epitaxial heterostructures. Although progress has been faster for lattice-matched systems, the additional degree of freedom for band

structure engineering offered by strain has become increasingly attractive. Nowadays, most state-of-the-art microprocessors exploit strained Si (3).

The strain introduced by growing a single-crystalline layer of one material on a second crystal differing in lattice parameter can persist only up to a certain critical thickness (4, 5). Beyond that thickness, segments of misfit dislocations form at the interface, gradually relieving the misfit strain as growth proceeds. Misfit dislocation segments are always accompanied by

threading dislocations extending to the surface (5). From a practical point of view, threading dislocations are most undesirable, because they may penetrate active device regions far away from a dislocated interface. Numerous methods have been more or less successful in reducing threading dislocation densities (6–18).

Equally fundamental problems arise for applications requiring thick layers, such as high-brightness light-emitting diodes, power transistors, or multiple-junction solar cells. Different thermal expansion coefficients of layers and substrates then often cause layer cracking (19) and wafer bowing (20), prohibiting further wafer processing or causing device failure. Regarding these effects, frequently dwarfing the dislocation problem in practical importance, no satisfactory solution has been found to date.

In the quest for a viable path toward the monolithic integration of an x-ray imaging detector onto

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a complementary metal oxide semiconductor (CMOS) chip, we have developed a solution that may pave the way for the hetero-epitaxial growth of mismatched structures of any vertical scale onto any substrate. We consider the case of very thick Ge and SiGe layers deposited in a maskless process on a patterned Si(001) substrate. For pure Ge, the lattice and thermal expansion coefficient mismatches at room temperature are $\sim 4.2\%$ (21) and $\sim 130\%$ (22), respectively. We show that continuous films can be prevented from forming by growing far from equilibrium onto substrate features with high aspect ratios (i.e., height to base width). This results in a uniform space-filling array of three-dimensional (3D) epitaxial crystals, coalescence of which is avoided by self-limited lateral expansion, whereas their height, size, and shape can be tuned over a wide range by growth and substrate parameters. The observed piling up of crystalline material, dictated by kinetics and geometry, thus mimics 3D growth of bulk ingots on a micrometer scale.

Figure 1A shows an example of Ge crystals, grown on a periodic array of Si seeds. The seeds were formed by deep micromachining of (001)-oriented (within $\pm 0.5^\circ$) Si substrates (23) into pillars from 1.7 to 8 μm in height, with base width from 0.75 to 15 μm and spacing from 200 nm to 50 μm . Scanning electron microscopy (SEM) images of 8- μm -tall and 2- μm -wide pillars are displayed in Fig. 1B. Subsequently, Ge was deposited epitaxially by low-energy plasma-enhanced chemical vapor deposition (LEPECVD) (24) at a high rate of 4 nm/s and growth temperatures T_G from 415° to 585°C (23). The SEM micrographs of Fig. 1C show arrays of Ge crystals grown on the two patterns of Fig. 1B. In contrast to the faceted crystals of Fig. 1A, they are shaped as flat-topped towers because of the lower growth temperature.

The deposition is highly nonconformal, characterized by self-limited lateral expansion near the bottom of the towers, subsequently evolving into vertical growth, irrespective of the amount of Ge material deposited and the details of the substrate patterns (Fig. 2A, and figs. S1 and S2) (23). The axes of the towers are defined by the growth direction, even when intentionally mis-

oriented substrates ($\sim 6^\circ$) are used (fig. S3). The general shape of the towers is practically unaffected by the surface finishing (clean or oxidized) of the Si pillars, their sidewall roughness, and their exact orientation with respect to [001] (figs. S3 to S5). It does not even depend on the lattice and thermal mismatch, as a comparison of pure Si, $\text{Si}_{0.6}\text{Ge}_{0.4}$ alloy and pure Ge towers shows (fig. S6).

Depending on the choice of substrate pattern and the amount of material deposited, the arrays visible in Fig. 1C can cover almost the entire substrate surface, resulting in space fillings of up to 96%. By preventing the formation of a continuous layer, crack propagation and wafer bowing are inhibited, no matter how thick the epitaxial deposition. This can be seen in the Nomarski interference contrast image of Fig. 2B, showing the boundary between patterned and unpatterned regions of a 27- μm -thick layer. The continuous region (blue) exhibits many cracks due to the thermal stress developed during cool-down from the growth temperature, but no cracks have propagated into the patterned region (gray). The insets show expanded Nomarski and SEM views of Ge towers, exhibiting an aspect ratio of about 5.

In order to assess the crystal quality, tilt, and strain status of the Ge towers we used high-resolution x-ray diffraction, with reciprocal space mapping around the Si(004) and Si(224) reflections (23). The results provided evidence for the nearly perfect crystal structure of the towers and showed that the Ge material on the Si pillars is completely unstrained (figs. S7 to S9). The full relaxation of the thermal strain in the case of deposition on patterned Si substrates is likely to be purely elastic, provided by the high aspect ratio of the Ge towers, as confirmed by finite element method simulations (fig. S10) (23).

In order to understand which particular deposition features generate the growth and self-limited lateral expansion of Ge towers observed in Figs. 1 and 2 and figs. S1 to S7, we set up a 2D growth model based on a rate equation for the adatom phase, commonly used for interpreting facet growth in selective area deposition (SAD) experiments (25, 26). In such an empirical mod-

el, the temporal variation of the adatom phase, which is usually a fraction of a monolayer, at a specific surface site is determined by the balance between the incoming flux, the incorporation rate into the crystal, the etching (or desorption) rate, and the diffusion from one site to another or from one facet to another (23).

The first key point in our experiments to be accounted for is the high deposition rate of 4 nm/s on (001) surfaces at low T_G (415° to 585°C). We estimate mean free paths for surface diffusion to be on the order of 100 to 200 nm in the time taken to deposit one monolayer, so that diffusion can be neglected on the scale of tower facets. Favored facets, typically {110}, {111}, {113}, and {001}, hence grow independently. The growth velocity perpendicular to a facet $\{hkl\}$ is $v = \chi \cdot \Phi$, where χ is an incorporation factor, representing the net fraction of the incoming flux Φ incorporated into the solid under stationary conditions. This factor is related to the balance between crystallization and etching/desorption rates, which depend on the facet considered.

In addressing the flux Φ on different facets, we take into account both the velocity distribution of the activated gaseous species in the LEPECVD deposition chamber (27) and the inclination of each facet. Because of the large (compared to the pattern size) mean free path for the scattered molecules in the gas phase (some centimeters for average chamber pressures of $\sim 10^{-2}$ mbar), the total flux can be described as the superposition of a dominant isotropic part Φ_1 , indicated by red arrows in Fig. 3A, and a uniform vertical contribution Φ_2 , accounting for the preferential motion induced by charged species propagating from the plasma source to the sample (green arrows in Fig. 3A) (27). Their ratio is set to approximately 3:1, in order to account for the experimental accumulation at the bottom of the trenches (Fig. 3D), when progressive closure of the tower spacing is simulated. As a result, each facet experiences a different incoming flux Φ because of its orientation with respect to the vertical direction (fig. S11A).

The second key point of our experimental configuration is the additional reduction in the

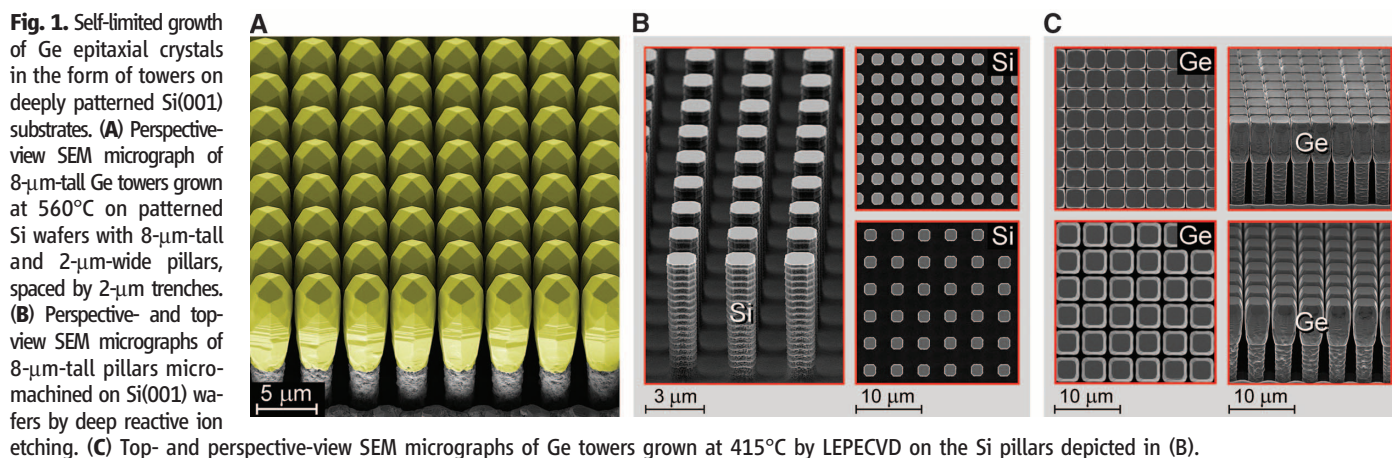


Fig. 1. Self-limited growth of Ge epitaxial crystals in the form of towers on deeply patterned Si(001) substrates. (A) Perspective-view SEM micrograph of 8- μm -tall Ge towers grown at 560°C on patterned Si wafers with 8- μm -tall and 2- μm -wide pillars, spaced by 2- μm trenches. (B) Perspective- and top-view SEM micrographs of 8- μm -tall pillars micro-machined on Si(001) wafers by deep reactive ion etching. (C) Top- and perspective-view SEM micrographs of Ge towers grown at 415°C by LEPECVD on the Si pillars depicted in (B).

incoming flux by the shielding from neighboring towers, so that it is no longer uniform across a facet. After initial vertical and lateral growth, the flux component Φ_1 on vertical $\{110\}$ facets is progressively reduced as the space between the Ge towers gets smaller (fig. S11B). This is accompanied by a progressive reduction of the lateral growth rate. As is well known from SAD literature (28), a facet extends in size when its growth velocity drops with respect to that of neighboring facets. To a lesser extent, the same happens to inclined $\{113\}$ and $\{111\}$ facets.

Figure 3B shows how the growth is simulated in a 2D model. We start from an initial distribution of $\{110\}$, $\{111\}$, $\{113\}$, and $\{001\}$ facets, provided by SEM images at the early stages of the deposition. The resulting surface profile is partitioned into independent segments of ~ 150 nm. Perpendicular growth in the time step δt is $v \cdot \delta t$, where $v = \chi \cdot \Phi$ as defined above. Φ varies smoothly across a facet, mainly because of mutual tower shielding, but a larger variation occurs at the vertex between differently oriented facets (angle α and β for facet I and II, respectively, in Fig. 3B). Here, the segments are elongated up to their crossing point, in order to simulate the active intrasegment diffusion. If such a vertex (purple arrow in the bottom inset of Fig. 3B) stays within the green area as growth proceeds, both facets expand evenly, whereas one of them shrinks while the other expands if the vertex moves into a yellow area. This geometric condition, expressed by the angle θ in Fig. 3B, depends on the growth velocity ratio of the two adjacent segments. Therefore, the incorporation factors for inclined facets can be estimated by tracing the vertex evolution from the experimental SEM profiles for a single isolated pillar at different deposition stages (for example, the dashed white lines in Fig. 3C). In that case, Φ is determined by the facet inclination only, because flux shielding is absent. The incorporation factors for the inclined facets with respect to χ_{001} are found to be $\chi_{113} = 0.96$, $\chi_{111} = 0.89$, and $\chi_{110} = 0.78$ (23).

We used these fitted χ_{hkl} values to calculate the extended growth profiles in Fig. 3C (white lines superimposed on colored experimental SEM images) around an isolated pillar at different growth stages. The overall agreement is very good, especially for coverage below $10 \mu\text{m}$.

By using the same χ_{hkl} values, and by taking into account the incoming flux shielding along the facets produced by nearby Ge towers, we simulated the shape evolution for a pattern with Si pillars spaced $4 \mu\text{m}$ apart. The excellent agreement between the experimental SEM profiles (in full color) and simulated (white lines) tower profiles (Fig. 3D and fig. S12) confirms the key role of geometric shielding. The vertical growth of the towers (see, for example, the SEM image in Fig. 2A) arises because the flux of activated gaseous species toward the $\{110\}$ facets drastically drops when the spacing between towers shrinks below some tens of nanometers (fig. S1).

The surface filling of closely spaced but isolated towers is remarkably stable with respect to changes of the pattern geometry and variations in the deposition conditions. The surface morphology of the Ge towers can, however, be modified by careful tuning of the kinetic conditions. This becomes apparent from Fig. 4, A to C,

showing SEM images of the substantial changes occurring with variations of T_G . Although the lowest T_G of 415°C (Fig. 4A) guarantees Ge towers with extended, flat $\{001\}$ tops, progressive faceting sets in with increasing T_G , culminating in full $\{113\}$ pyramidal tops at $T_G = 585^\circ\text{C}$ (Fig. 4C).

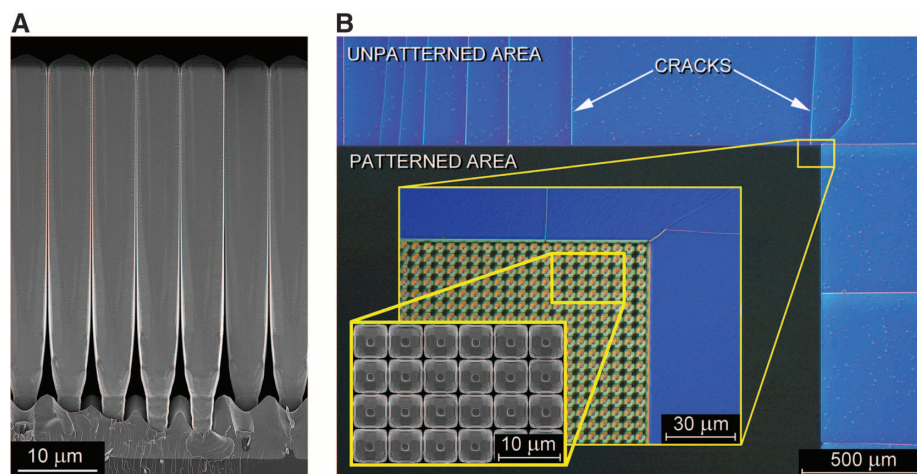


Fig. 2. Crack inhibition in a Ge/Si(001) system. (A) SEM micrograph of 50- μm -tall Ge towers, obtained by self-limited lateral growth at 490°C on a patterned Si substrate with 8- μm -tall and 2- μm -wide pillars, spaced by 4- μm trenches. (B) Nomarski interference contrast micrographs of 27- μm -tall Ge towers grown at 490°C on patterned Si substrates. Crack propagation stops at the border between patterned and unpatterned areas. The inset in the lower left corner is a top-view SEM micrograph of a few Ge towers.

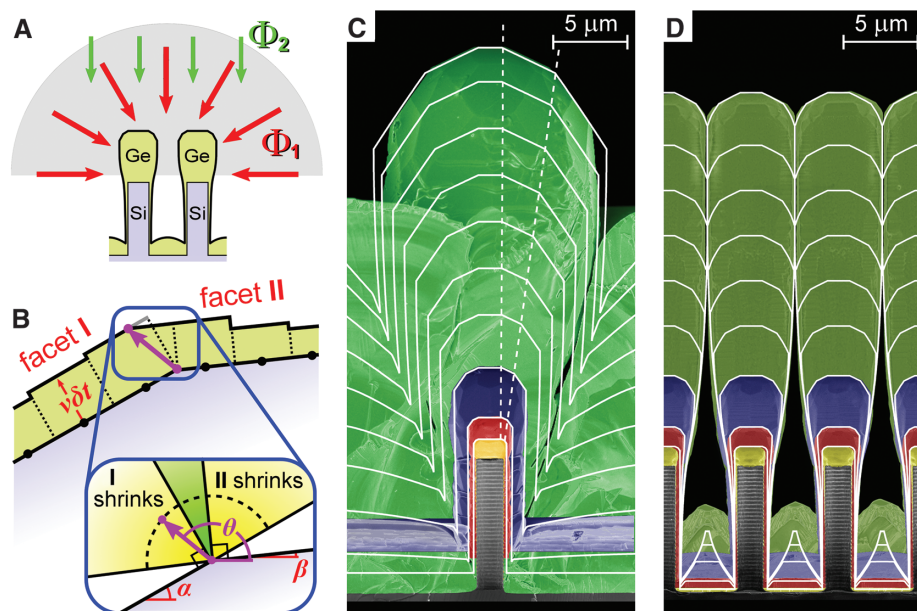


Fig. 3. Modeling of self-limited Ge tower growth. (A) Isotropic (red arrows) and vertical (green arrows) contributions to the incoming flux. Facet orientation and mutual shadowing among towers determine the effective flux at each surface region. (B) Sketch of a simulated profile at an edge between two neighboring facets, evolving in a time step δt . The facets are partitioned into segments, each one growing at its own velocity. Edges (purple arrow) evolve according to the ratio between facet growth velocities, as indicated in the inset, establishing a relation between geometric evolution and kinetic parameters. (C) The experimental facet evolution (colored SEM image) of an isolated Ge crystal grown at 490°C is used to fit the parameters of the model, yielding the profiles indicated by white lines at different growth stages. The edges between $\{001\}$ and $\{113\}$, and $\{113\}$ and $\{111\}$ facets are indicated by dashed lines. (D) Simulated profile evolution (white lines) superimposed on experimental SEM images (full color) of Ge towers grown at 490°C on a patterned substrate with 2- μm -wide Si pillars, spaced by 4- μm trenches. In (C) and (D), the various colors correspond to different samples with different deposited thicknesses.

In the growth model outlined above, giving rise to simulated surface profiles in very good agreement with those seen experimentally (Fig. 3D), $v = \chi \cdot \Phi$ does not depend explicitly on temperature. The incorporation rate χ of each facet may, however, depend on T_G , as certainly does the surface diffusion between neighboring facets. A small amount of unbalanced surface flux can therefore result, provided that the facets differ favorably in terms of surface energy or adatom density. In order to estimate this effect, the ratio v_{113}/v_{001} of facet growth velocities was calculated from the temperature variation of the experimental profiles. The resulting ratios, displayed above the SEM images in Fig. 4, A to C, may be seen to vary only between 0.92 and 0.86 from the lowest to the highest T_G . This shows that slight variations of the relative velocities may cause drastic changes of the surface morphology.

Finally, we want to emphasize the key role of the tower morphology in controlling the threading dislocation density. The 60° dislocations present

in crystals with the zincblende and diamond structures (5, 6, 29) have threading arms gliding in $\{111\}$ planes and are directed along $\langle 110 \rangle$. As indicated in the schematic drawing of Fig. 4D and in the cross-section transmission electron microscopy (TEM) images to its right, threading dislocations that are not parallel to $[001]$ are geometrically expelled at the edges of 4- μm -wide and 8- μm -tall towers. Similar observations have been made in much smaller structures fabricated by SAD into dielectric windows on flat, unpatterned substrate surfaces exhibiting sufficiently large aspect ratios (13, 15). This method is therefore also known by the name aspect ratio trapping (ART) (15). In contrast to experiments on ART, where the thickness of the dielectric and the submicrometer pattern size govern the attainable aspect ratio, this quantity may be tuned by varying the tower height in our case. In flat-top towers of the kind visible in the SEM images of Fig. 4A, additional dislocations may be present, oriented approximately along $[001]$.

Examples of these so-called growth dislocations (14, 30, 31), remaining trapped along the entire tower height, may be seen in the TEM images in the lower right of Fig. 4D. Because cross-section TEM is poorly suited for an analysis of the threading dislocation density, we additionally used the method of etch pit counting. Indeed, an atomic force microscopy (AFM) analysis performed after defect etching revealed evenly distributed etch pits on the (001) top facets (upper right of Fig. 4D). Growth dislocations can only be expelled by surface faceting during growth (14), because they bend into directions perpendicular to a facet (see the schematic in Fig. 4E). This was shown to also be true for the submicrometer structures used in ART (16). We have demonstrated that the surface morphology of Ge towers can be controlled by adjusting T_G (Fig. 4, A to C). In the fully faceted towers obtained at higher T_G , the defects are indeed confined to the bottom part, according to the TEM images of Fig. 4E. The removal of threading dislocations from faceted Ge towers is confirmed by the absence of etch pits in AFM images such as that shown on the upper right of Fig. 4E.

The patterning of single-crystalline substrates into micrometer-sized features separated by deep trenches, combined with epitaxial deposition at a high rate, can lead to closely spaced crystals of arbitrary height by a mechanism of self-limiting lateral growth, thus mimicking the 3D growth of bulk ingots on a micrometer scale. We have shown for the Ge/Si(001) and SiGe/Si(001) systems that such a behavior is driven by geometric shielding of the incoming particle flux, combined with limited surface diffusion. Faceted Ge and SiGe crystals are dislocation-free and completely relaxed, despite the large lattice and thermal mismatch with the Si substrate. By avoiding the formation of continuous layers, wafer bowing and crack formation are inhibited. Although we have provided the proof of concept only for group IV semiconductors, we believe this new mode of hetero-epitaxial crystal growth to be applicable to most materials combinations used for the fabrication of semiconductor devices. We expect this to pave the way for many applications requiring thick hetero-epitaxial layers, such as x-ray and particle detectors monolithically integrated onto CMOS substrates, and power electronic devices or multijunction solar cells fabricated on cheap Si wafers.

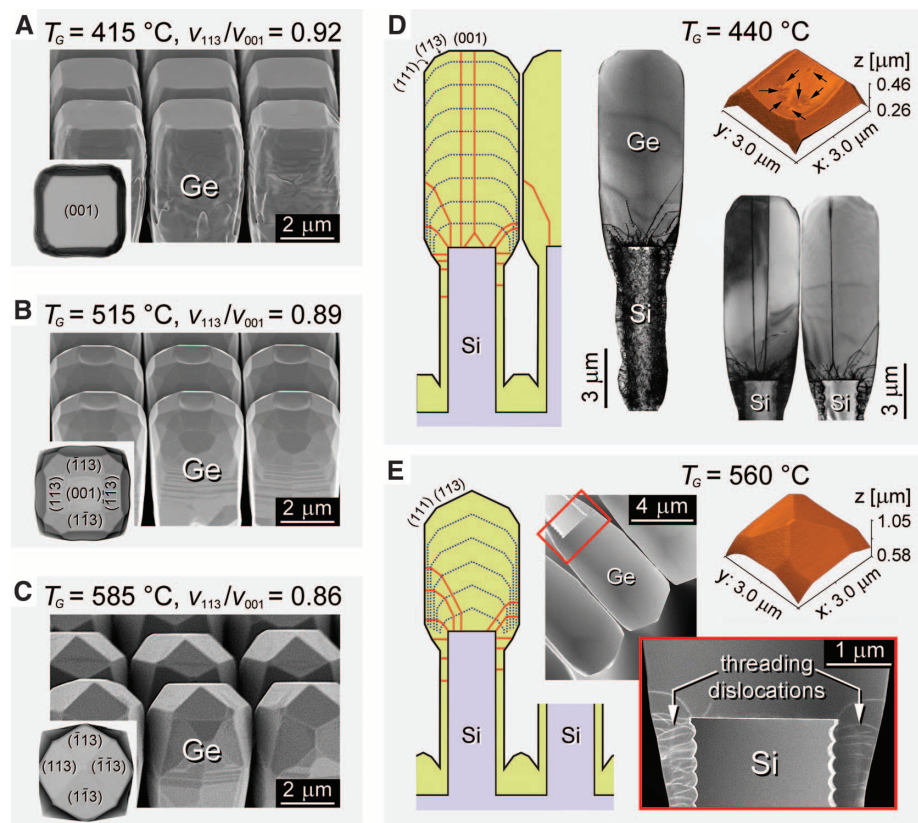


Fig. 4. Removal of threading dislocations by temperature-controlled faceting of the Ge towers. Shown are SEM micrographs of the surface morphology of Ge crystals evolving with increasing T_G , with flat tops at 415°C (A), partly faceted tops at 515°C (B), and full pyramidal shape at 585°C (C). The corresponding v_{113}/v_{001} velocity ratios derived from top-view and cross-sectional SEM micrographs vary from 0.92 to 0.86, as indicated. (D) (Left) Schematic drawing of growth dislocations trapped in Ge towers with (001) top facets, and of inclined dislocations escaping to the side walls. (Center) Brightfield TEM cross-section image acquired in the $[220]$ Bragg condition with inclined dislocations only. (Right) Corresponding TEM images with both kinds of dislocations, and an AFM micrograph taken after defect etching in an iodine solution. Etch pits are indicated by black arrows. (E) Schematic drawing (left), darkfield STEM images acquired along the $[110]$ zone axis (middle), and AFM micrograph (right) for Ge crystals with only (113) and (111) facets. All dislocations are expelled from the crystals in this case, as confirmed by the absence of etch pits in the AFM image.

References and Notes

1. V. Umansky *et al.*, *J. Cryst. Growth* **311**, 1658 (2009).
2. H. L. Störmer, D. C. Tsui, *Science* **220**, 1241 (1983).
3. S. E. Thompson *et al.*, *IEEE El. Dev. Lett.* **25**, 191 (2004).
4. J. H. van der Merwe, *J. Appl. Phys.* **34**, 123 (1963).
5. J. W. Matthews, S. Mader, T. B. Light, *J. Appl. Phys.* **41**, 3800 (1970).
6. J. W. Matthews, A. E. Blakeslee, S. Mader, *Thin Solid Films* **33**, 253 (1976).
7. P. D. Hodson *et al.*, *Semicond. Sci. Technol.* **3**, 715 (1988).
8. E. A. Fitzgerald *et al.*, *Appl. Phys. Lett.* **59**, 811 (1991).
9. E. A. Fitzgerald, N. Chand, *J. Electron. Mater.* **20**, 839 (1991).
10. For a review of early work, see also (31).
11. M. R. Goulding, *Mater. Sci. Eng. B* **17**, 47 (1993).
12. H. C. Luan *et al.*, *Appl. Phys. Lett.* **75**, 2909 (1999).

13. T. A. Langdo *et al.*, *Appl. Phys. Lett.* **76**, 3700 (2000).
14. H. Klapper, *Mater. Chem. Phys.* **66**, 101 (2000).
15. J.-S. Park *et al.*, *Appl. Phys. Lett.* **90**, 052113 (2007).
16. J. Bai *et al.*, *Appl. Phys. Lett.* **90**, 101902 (2007).
17. S. Hu, P. W. Leu, A. F. Marshall, P. C. McIntyre, *Nat. Nanotechnol.* **4**, 649 (2009).
18. G. Wang *et al.*, *Appl. Phys. Lett.* **96**, 111903 (2010).
19. E. Feltn *et al.*, *Appl. Phys. Lett.* **79**, 3230 (2001).
20. M. Sakai, T. Egawa, M. Hao, H. Ishikawa, *Jpn. J. Appl. Phys.* **43**, 8019 (2004).
21. Y.-W. Mo, D. E. Savage, B. S. Swartzentruber, M. G. Lagally, *Phys. Rev. Lett.* **65**, 1020 (1990).
22. G. A. Slack, S. F. Bartram, *J. Appl. Phys.* **46**, 89 (1975).
23. Materials and methods and supporting text are available on Science Online.
24. C. Rosenblad *et al.*, *J. Vac. Sci. Technol. A* **16**, 2785 (1998).
25. M. Ohtsuka, S. Miyazawa, *J. Appl. Phys.* **64**, 3522 (1988).
26. S. Li, Q. Xiang, D. Wang, K. L. Wang, *J. Cryst. Growth* **157**, 185 (1995).
27. M. Rondanini *et al.*, *J. Appl. Phys.* **104**, 013304 (2008).
28. S. H. Jones, L. K. Seidel, K. M. Lau, M. Harold, *J. Cryst. Growth* **108**, 73 (1991).
29. E. P. Kvam, D. M. Maher, C. J. Humphreys, *J. Mater. Res.* **5**, 1900 (1990).
30. G. R. Booker *et al.*, *J. Cryst. Growth* **45**, 407 (1978).
31. A. Madhukar, *Thin Solid Films* **231**, 8 (1993).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/335/6074/1330/DC1
Materials and Methods
SOM Text
Figs. S1 to S12
References (32–36)

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A Change in the Geodynamics of Continental Growth 3 Billion Years Ago

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Models for the growth of continental crust rely on knowing the balance between the generation of new crust and the reworking of old crust throughout Earth's history. The oxygen isotopic composition of zircons, for which uranium-lead and hafnium isotopic data provide age constraints, is a key archive of crustal reworking. We identified systematic variations in hafnium and oxygen isotopes in zircons of different ages that reveal the relative proportions of reworked crust and of new crust through time. Growth of continental crust appears to have been a continuous process, albeit at variable rates. A marked decrease in the rate of crustal growth at ~3 billion years ago may be linked to the onset of subduction-driven plate tectonics.

The timing, rates, and the geodynamical conditions of continental crust generation, destruction, and reworking remain a topic of considerable debate (1–7). The variations in radiogenic isotope ratios in detrital rocks and minerals are a key archive of the continental record (3–5, 8), and the rapid increase in the numbers of U-Pb and Hf isotope analyses of predominantly detrital zircons have provided new constraints for models of crustal evolution (4). Hf isotopes in U-Pb-dated zircons are commonly used to characterize the nature of the source of the magma from which the zircon crystallized and to determine the time since this source separated from the upper mantle, commonly referred to as the model age of crust formation (9–11). However, individual model ages may not represent true periods of crust formation (12) because the crustal material analyzed may represent mixtures of older “reworked” and new

juvenile material. Continental growth models based simply on U-Pb and Hf isotopes in zircon therefore have a large uncertainty over the proportions of new continental crust generated in different magmatic episodes, and hence over the shape of the continental growth curve (4).

Combining stable isotopes, such as oxygen, with the radiogenic isotopes of U-Pb and Lu-Hf (6, 13) may reduce uncertainties surrounding the proportion of new and reworked crust. “Mantle-like” zircons, that is, zircons that crystallized from mantle-derived magmas, have a narrow range of $^{18}\text{O}/^{16}\text{O}$ (expressed as $\delta^{18}\text{O}$ relative to Vienna standard mean ocean water), typically $\delta^{18}\text{O} = 5.3 \pm 0.6\%$ (per mil) (2 SD) (14). When their parent magmas contain a contribution of sedimentary material or source rocks altered by low- (or high-) temperature hydrothermal activity, the $\delta^{18}\text{O}$ in zircons can range to higher (or lower) values (15). In principle, periods of juvenile crust formation should be characterized by zircons with mantle-like $\delta^{18}\text{O}$ and similar radiogenic Hf model ages (6). Conversely, periods dominated by crustal reworking result in the generation of “supracrustal” zircons, typically with elevated $\delta^{18}\text{O}$ values and varying Hf model ages (13, 15, 16). To the extent that Hf isotope ratios of supracrustal zircons represent mixtures, they will not record

true periods of crustal growth (6). There are relatively few studies in which U-Pb and Hf isotopes are combined with O isotopes in zircon (6, 7, 17, 18), and evaluating crustal growth models based on large data sets of zircons remains difficult, especially when constraining the proportions of new crust formation ages to those that are arguably hybrid ages (4, 8, 19).

$\delta^{18}\text{O}$ values are plotted as a function of Hf model ages in 1376 detrital and inherited zircons from Australia, Eurasia, North America, and South America (Fig. 1A) (17). These data are taken to be representative of the Hf-O isotope record available for Earth's continental crust. Overall supracrustal zircons with Meso/Paleo-Proterozoic Hf model ages show a greater range of $\delta^{18}\text{O}$ values than those with older or younger model ages, and they are more abundant than mantle-like zircons. The proportions of mantle-like zircons and supracrustal zircons in turn determines the relative proportions of new crust formation ages and hybrid model ages induced by crustal reworking processes in the distribution of Hf model ages (Fig. 1B). The proportion of new crust formation ages does not change substantially in the first billion years of Earth's history, with a median value ~73%. From ~3.2 billion years ago (Ga), the proportion of new crust formation ages gradually decreases down to ~20% at ~2 Ga, and it then increases to ~100% toward the present day (Fig. 1B). Assuming that these observations characterize the continental crust as a whole, this parameterization allows us to predict the proportion of new crust and hybrid model ages throughout Earth's evolution and, hence, access the large U-Pb and Hf isotope data sets that do not include $\delta^{18}\text{O}$ data (4, 8, 19).

Young sediments typically contain zircons with a wide range of ages, so they appear to provide records that are more representative of the magmatic history of the crust than zircons in igneous rocks or in old sediments (5). A compilation of 6972 analyses of detrital zircons with deposition ages ranging from the late Paleozoic to the present day (11) results in a distribution of Hf model ages that does not simply reflect the generation of new crust, because it still includes

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Fig. 1. (A) $\delta^{18}\text{O}$ versus Hf model ages in 1376 detrital and inherited zircons (11) from Australia [this study, $n = 458$; and (6), $n = 106$], Eurasia (18, 29) ($n = 488$), North America (7, 17) ($n = 232$), and South America (30) ($n = 92$). Zircons with $\delta^{18}\text{O}$ within error of the mantle-like zircons domain [$\delta^{18}\text{O} = 5.3 \pm 0.6\text{‰}$, 2 SD (14)] are referred to as mantle-like zircons. Their Hf model ages record periods when new crust is generated. Other zircons are referred to as supracrustal. Their Hf model ages are referred to as “hybrid,” because they may not record true periods of new crust formation. Model ages are calculated using the reference line for the Hf isotope evolution of the new crust of (9) and $^{176}\text{Lu}/^{177}\text{Hf} = 0.015$ for the crustal source. The calculated model ages are sensitive to the values chosen, but the shape of the age distributions are much less so (figs. S1 and S2). **(B)** O isotopes are used to distinguish hybrid model ages (grey bins, supracrustal zircons data) from model ages that represent periods of new crust generation (green bins, mantle-like zircon data). The data points represent the proportion of Hf model ages associated with new crust generation in the worldwide zircon record, calculated for 100-million-year time intervals, and when there are $n \geq 3$ analyses for each time interval. The red and brown dots define a relationship through time (black curve) that is defined by Eqs. 1 and 2, respectively (the white-filled red dot was not included in the calculation of Eq. 1).

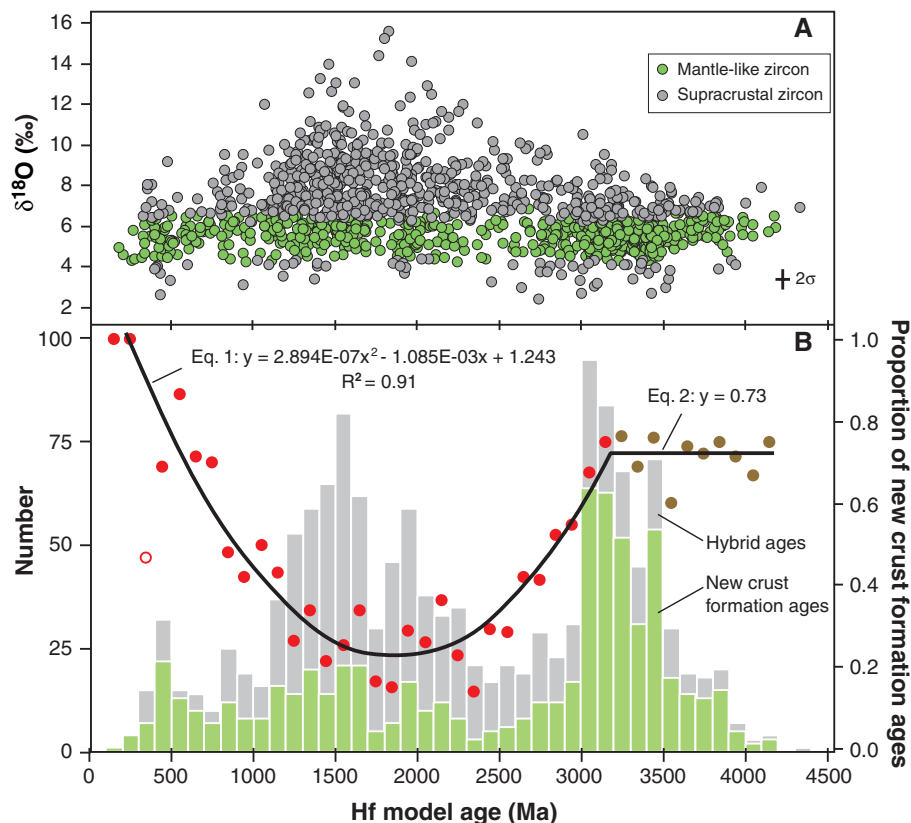
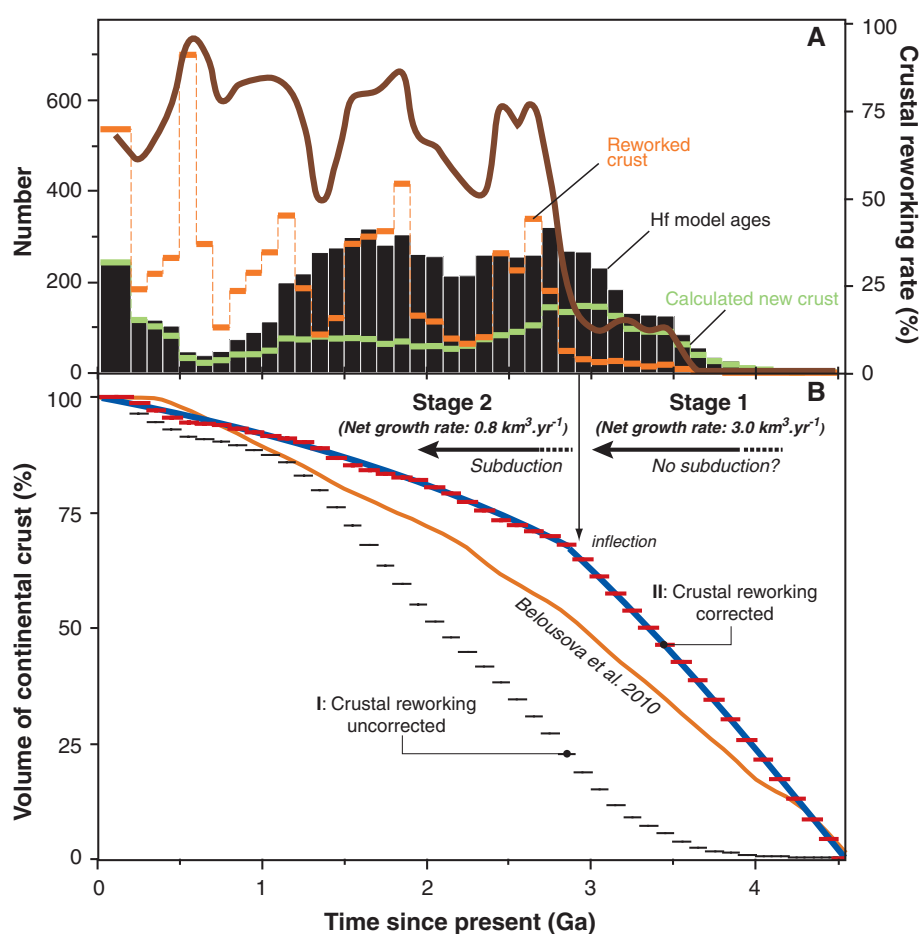


Fig. 2. (A) Distribution of the Hf model ages, calculated new crust ages, and reworked crust ages through time, from a worldwide database of 6972 U-Pb and Hf analyses of zircons from young sediments, with deposition ages ranging from the late Paleozoic to the present day (11). All calculations are presented for every 100-million-year time interval starting from 200 million years ago (Ma). The youngest bin is for 200 Ma because of uncertainty over the range of deposition ages of the sediments. The brown curve represents the variation in the rates of reworking of the continental crust through time, calculated from the distributions of the proportions of reworked crust (orange histogram) and new crust (green histogram). **(B)** Continental growth curves calculated from the same database as (A). The cumulative volume of crust is calculated for 100-million-year time intervals. Curve I (black) is calculated from the cumulative proportions of the Hf model ages through time [black histogram in (A)]. Curve II (red) integrates the variations of the reworking rates [brown curve in (A)] in the calculations of the cumulative proportions of the newly formed crust through time. The blue curve highlights the main variations observed in curve II. The overall shape of this curve is not substantially affected by the selection of the Lu/Hf ratios in the crust and the Hf isotope ratios of new continental crust in the calculation of individual model ages (figs. S1 and S2). The curve obtained by Belousova *et al.* (4) from a U-Pb-Hf database of 13,844 zircons of various ages and origin is reported for comparison.



hybrid model ages (Fig. 2A). However, using these data, and our relation that characterizes the proportion of new crust and hybrid model ages through time (Fig. 1B, Eqs. 1 and 2), we were able to calculate the distribution of new crust formation ages (Fig. 2A).

The net volume of new continental crust generated during magmatic episodes depends on the variations in the proportion of newly formed and reworked crust that is preserved through time. A broader proxy for the variations of the reworked crust through time is given by the distribution of the crystallization ages of zircons with Hf model ages greater than their crystallization ages (Fig. 2A). These variations may be linked to times of supercontinent assembly, periods that are characterized by both increased crustal reworking and preservational bias (8, 20, 21). The variation in the rates of crustal reworking through time, which is given by the variations in the proportions of the reworked crust versus calculated new crust, reveals low crustal reworking rates (< 20%) from the Hadean to the Meso-Archean. A rapid increase to around 75% reworking is observed at ~3 Ga, and then relatively high reworking rates (>50%) are observed until the present day.

This parameterization has several implications for models of the rates of growth of the continental crust (Fig. 2B). The distribution of Hf model ages, irrespective of their oxygen isotope ratios and the extent to which they include hybrid model ages (Fig. 2A), provides an estimate of the minimum volume of the preserved continental crust that was present through time, because it does not integrate the variations in the proportions of reworked and new crust. If, however, the model integrates crustal generation and reworking rates (Fig. 2A), we see that ~65% of the present-day volume of the crust was established by 3 Ga (Fig. 2B) and that there was a sharp change in the net rates of growth of the continental crust at that time. These features are not observed in continental growth curves calculated from large U-Pb and Hf in zircon databases in the absence of O isotope data (4).

Based on this analysis, we propose a two-stage model for continental growth (Fig. 2B). Stage 1 (>4 Ga to ~3 Ga) is characterized by relatively high net rates of continental growth. Given the present-day volume of the continental crust of 7.10^9 km^3 [e.g., (22)], the average net rate of growth of the continental crust for Stage 1 was $\sim 3.0 \text{ km}^3 \text{ year}^{-1}$. This is similar to the rates at which new crust is generated and destroyed at the present time (22, 23). The average net growth rate for Stage 2 (~3 Ga to the present day) is $\sim 0.8 \text{ km}^3 \text{ year}^{-1}$, and the inflection in the rate of crustal growth curve at ~3 Ga indicates a fundamental change in the way the continental crust was generated and preserved. The difference in the net rates of crustal growth in Stages 1 and 2 can be accommodated by high rates of destruction of continental crust in Stage 2 compared with Stage 1 (Fig. 2A). The inferred high crustal destruction rates in Stage 2 strongly

suggest that it reflects the onset of subduction-driven plate tectonics and discrete subduction zones at ~3 Ga, consistent with independent arguments from recent studies (24–26). Geodynamical processes that might have dominated in Stage 1 include shallow subduction and delamination (27) or “intraplate” lithospheric extension/mantle upwelling (26, 28), both of which could produce crust at rates similar to today ($\sim 3.0 \text{ km}^3 \text{ year}^{-1}$).

References and Notes

- K. C. Condie, *Earth Planet. Sci. Lett.* **163**, 97 (1998).
- B. Dhuime, C. J. Hawkesworth, C. D. Storey, P. A. Cawood, *Geology* **39**, 407 (2011).
- C. J. Allègre, D. Rousseau, *Earth Planet. Sci. Lett.* **67**, 19 (1984).
- E. A. Belousova *et al.*, *Lithos* **119**, 457 (2010).
- C. J. Hawkesworth *et al.*, *J. Geol. Soc. London* **167**, 229 (2010).
- A. I. S. Kemp, C. J. Hawkesworth, B. A. Paterson, P. D. Kinny, *Nature* **439**, 580 (2006).
- C. Y. Wang, I. H. Campbell, C. M. Allen, I. S. Williams, S. M. Eggins, *Geochim. Cosmochim. Acta* **73**, 712 (2009).
- K. C. Condie, M. E. Bickford, R. C. Aster, E. Belousova, D. W. Scholl, *Geol. Soc. Am. Bull.* **123**, 951 (2011).
- B. Dhuime, C. J. Hawkesworth, P. A. Cawood, *Science* **331**, 154 (2011).
- D. J. DePaolo, *Nature* **291**, 193 (1981).
- Materials and methods are available as supporting material on Science Online.
- N. T. Arndt, S. L. Goldstein, *Geology* **15**, 893 (1987).
- C. J. Hawkesworth, A. I. S. Kemp, *Chem. Geol.* **226**, 144 (2006).
- J. W. Valley, P. D. Kinny, D. J. Schulze, M. J. Spicuzza, *Contrib. Mineral. Petrol.* **133**, 1 (1998).
- J. W. Valley *et al.*, *Contrib. Mineral. Petrol.* **150**, 561 (2005).
- A. I. S. Kemp *et al.*, *Science* **315**, 980 (2007).
- A. B. Pietranik *et al.*, *Geology* **36**, 875 (2008).
- C. Y. Wang, I. H. Campbell, A. S. Stepanov, C. M. Allen, I. N. Burtsev, *Geochim. Cosmochim. Acta* **75**, 1308 (2011).
- P. J. Voice, M. Kowalewski, K. A. Eriksson, *J. Geol.* **119**, 109 (2011).
- I. H. Campbell, C. M. Allen, *Nat. Geosci.* **1**, 554 (2008).
- C. J. Hawkesworth, P. A. Cawood, T. Kemp, C. Storey, B. Dhuime, *Science* **323**, 49 (2009).
- D. W. Scholl, R. von Huene, in *Earth Accretionary Systems in Space and Time*, P. A. Cawood, A. Kröner, Eds. (Geological Society, London, Special Publications, 2009), vol. 318, pp. 105–125.
- D. W. Scholl, R. von Huene, in *4-D Framework of Continental Crust*, R. D. Hatcher, M. P. Carlson, J. H. McBride, J. R. M. Catalán, Eds. (Geological Society of America Memoir, 2007), vol. 200, pp. 9–32.
- P. A. Cawood, A. Kröner, S. Pisarevsky, *GSA Today* **16**, 4 (2006).
- S. B. Shirey, S. H. Richardson, *Science* **333**, 434 (2011).
- M. J. Van Kranendonk, *Science* **333**, 413 (2011).
- S. F. Foley, S. Buhre, D. E. Jacob, *Nature* **421**, 249 (2003).
- M. J. Van Kranendonk, *Am. J. Sci.* **310**, 1187 (2010).
- P. J. Lancaster, C. D. Storey, C. J. Hawkesworth, B. Dhuime, *Earth Planet. Sci. Lett.* **305**, 405 (2011).
- C. W. Rapela *et al.*, *Gondwana Res.* **20**, 673 (2011).

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Materials and Methods

SOM Text

Figs. S1 and S2

References (31–72)

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The Multielectron Ionization Dynamics Underlying Attosecond Strong-Field Spectroscopies

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Subcycle strong-field ionization (SFI) underlies many emerging spectroscopic probes of atomic or molecular attosecond electronic dynamics. Extending methods such as attosecond high harmonic generation spectroscopy to complex polyatomic molecules requires an understanding of multielectronic excitations, already hinted at by theoretical modeling of experiments on atoms, diatomics, and triatomics. Here, we present a direct method which, independent of theory, experimentally probes the participation of multiple electronic continua in the SFI dynamics of polyatomic molecules. We use saturated (*n*-butane) and unsaturated (1,3-butadiene) linear hydrocarbons to show how subcycle SFI of polyatomics can be directly resolved into its distinct electronic-continuum channels by above-threshold ionization photoelectron spectroscopy. Our approach makes use of photoelectron-photofragment coincidences, suiting broad classes of polyatomic molecules.

To date, measurements of attosecond electronic dynamics in atoms and molecules (1) have typically involved strong laser-field processes. Even pump-probe spectroscopies using isolated attosecond pulses rely on the presence of a phased, strong ($\sim 10^{13} \text{ W/cm}^2$) laser field (2–4). The process of high harmonic gen-

eration (HHG), which can probe electronic wave packets on attosecond time scales (5–9), is entirely based on strong-field ionization (SFI), as is the use of subcycle electron recollision for probing dynamics (10, 11). In the interpretation of experiments, broad use is made of the three-step model (12) wherein SFI adiabatically releases, via

tunneling, a single continuum electron that is subsequently driven in the strong, oscillating laser field. However, even in simple diatomics, the possible role of multiple electronic continua in attosecond SFI measurements (5, 13, 14) is unclear (6, 15). In the complex world of multielectron polyatomic molecules, adiabatic single-electron tunneling approximations are very unlikely to hold (16–19). Nevertheless, to have broader impact, attosecond HHG spectroscopies must be extended to polyatomic molecules (20), and therefore, a general understanding of the role of multielectron SFI dynamics in polyatomics is required. Here, we introduce the channel-resolved above-threshold ionization (CRATI) method, where the channel refers to the electronic state of the ion correlated with the continuum electron. Independent of theoretical models, we experimentally resolve the multiple electronic continuum channels in SFI of polyatomic systems. By studying the CRATI of the linear hydrocarbons 1,3-butadiene and *n*-butane, we unambiguously demonstrate the direct, subcycle participation of multiple final ionic states in molecular SFI. We show that the population of higher-lying electronic continua can, depending on molecular properties, even exceed that of the ground state. We independently corroborate our experimental results with high-level, all-electron dynamical Schrödinger equation calculations. These combined results show that the relative contributions of these multiple continuum channels depend on both molecular electronic structure and laser-field properties and that both adiabatic and nonadiabatic laser-driven electronic dynamics may result.

A seminal attosecond transient absorption spectroscopy study recently demonstrated the coherent population (amplitudes and phases) of spin-orbit states due to SFI of krypton atoms (2). This technique benefitted from the presence of sharp features in the absorption spectrum that identified the two coherently prepared ionic states. To discern the ionic states of polyatomic molecules populated by SFI, alternate approaches might be required, because transient absorption spectroscopy of polyatomics is much more complex. Our experimental approach is based on above-threshold ionization (ATI) (21–24), a universal SFI phenomenon wherein a field-driven continuum electron absorbs excess photons beyond the minimum number required for ionization. This leads to observation of a series of peaks in the electron kinetic energy spectrum, spaced by the photon energy. There is a deep connection between ATI and the process of HHG. In

both cases, subcycle SFI generates a continuum electron that, depending on phase, either recollides with its ion core and emits a burst of extreme ultraviolet radiation (HHG) or accelerates away from the ion as an energetic electron (ATI). In either case, a single-cycle pulse would result in structureless HHG and ATI spectra. Structured spectra arise from this subcycle emission (photon or electron) being repeated every optical cycle. First evidence for the participation of multiple continua in HHG spectroscopy of molecules emerged from experiments on CO₂ (5, 25), N₂ (6, 26, 27), and N₂O₄ (28), each relying strongly on theoretical modeling for interpretation. These results caution that essential multiple continuum effects should not be ignored. Such effects are to be expected in the SFI of polyatomic molecules, because their many valence electrons should all respond to the strong laser electric field. We find, for the molecules studied here, that electrons can be ejected by tunneling via independent channels, populating the ionic ground state [ejection of the highest occupied molecular orbital (HOMO) electron;

i.e., HOMO ionization] and/or excited (for instance, HOMO–1 ionization) electronic states. By independent, we mean that the continuum channels remain uncoupled (that is, no transitions between them) in the laser field. However, in other molecular systems and/or at higher laser intensity, nonadiabatic interchannel coupling can occur during SFI. In this situation, another bound electron makes a laser-induced transition on a subcycle time scale as the continuum electron departs. This process is termed nonadiabatic multielectron (NME) ionization (16) and will be discussed below. Whether the response is adiabatic or not, population of multiple electronic continua will be a generic feature of SFI of polyatomic molecules. To distinguish SFI channels populating the ionic ground-state continuum from those populating ionic excited-state continua, we make use of the fact that, unlike diatomics, electronically excited polyatomic ions generally undergo rapid radiationless transitions, which leads to fragmentation (29, 30). This is the case for the linear hydrocarbons studied here, 1,3-butadiene (C₄H₆) and *n*-butane (C₄H₁₀):

Table 1. Vertical field-free ionization potentials I_p^F and Stark-shifted ionization potentials I_p^S (in electron volts) of the first four ionic states (D_j , $j = 0$ to 3) of *n*-butane and three ionic states (D_j , $j = 0, 1, 2$) of 1,3-butadiene. The I_p^F are measured values taken from (38) unless otherwise indicated. I_p^S values include the calculated Stark shift of the j th ionization potential, as described in the SOM. As the CRATI measurements were performed in presence of the laser field, the I_p^S values pertain for comparison with experiment.

Ionic state	Ionization potential	<i>n</i> -Butane	1,3-Butadiene
D_0	I_p^F	11.2	9.29*
	I_p^S	11.0	9.2
D_1	I_p^F	11.7	11.48*
	I_p^S	11.4	11.5
D_2	I_p^F	11.7	12.2
	I_p^S	11.5	11.7
D_3	I_p^F	12.2	(Not used)
	I_p^S	12.2	

*Taken from (39)

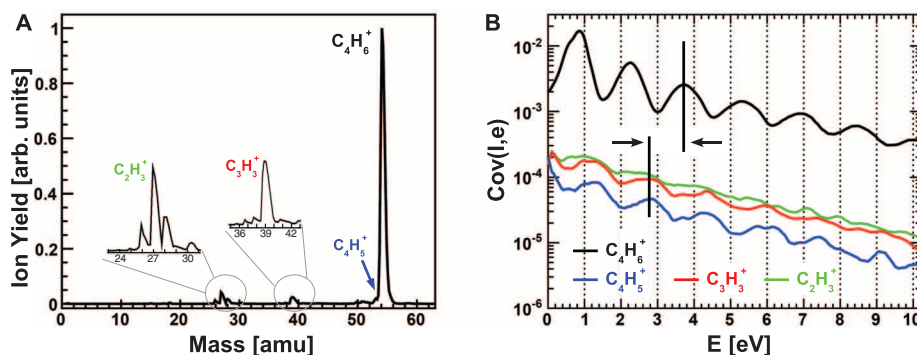


Fig. 1. Strong-field ionization of 1,3-butadiene at a peak intensity of 1.9×10^{13} W/cm². (A) Mass spectrum with the most prominent peak being the parent C₄H₆⁺ ion. Fragment ions C₄H₅⁺, C₃H₃⁺, and C₂H₃⁺ that originate from the unstable D₁ excited ion state were also observed. amu, atomic mass units. (B) CRATI photoelectron spectra correlated with both parent and fragment ions. As indicated by the vertical lines, the fragment CRATI combs are shifted in energy relative to that of the parent ion (black) by 0.95 ± 0.05 eV. This is quantitatively the energy difference between the Stark-shifted D₀ and D₁ ionic states in 1,3-butadiene, modulo the photon energy, which unambiguously demonstrates that the excited D₁ ion state was directly populated from the neutral ground state. For a detailed discussion, see the text.

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Only their ionic electronic ground state is stable, whereas transitions to electronically excited ionic states lead to unimolecular dissociation of the parent ion, producing fragments on a time scale much longer than the laser interaction (31–33). However, in a strong laser field, fragment ions may also be produced by postionization excitation of the ionic ground state. Therefore, the observation of ionic fragmentation in itself does not constitute proof that the SFI process directly populates electronically excited ionic states. Experimentally, we need to distinguish two SFI pathways: (i) subcycle ionization directly into an ionic excited state versus (ii) ionization forming the ionic ground state, followed by subsequent excitation during the laser cycles after ionization. In our CRATI experiment, this is achieved by measuring the ATI kinetic energy spectrum in coincidence (or covariance) with parent and fragment ions. As described in the supporting online material (SOM), the covariance-mapping method reveals correlations between ions and electrons via statistical analysis of count-rate fluctuations (34). The ATI spectrum consists of a comb of electron kinetic energies spaced by the photon energy (Eq. 1)

$$E = m\hbar\omega - [I_p^{(D_j)} - U_p] \quad (1)$$

where m is an integer and $\hbar\omega$ is the photon energy. For the molecular core, $I_p^{(D_j)}$ is the Stark-shifted molecular ionization potential of the j th ionization channel (where $j = 0$ signifies the D_0 ground electronic state of the ion, $j = 1$ the D_1 first excited electronic state of the ion, and so forth) and accounts for the relative Stark shifts of both neutral and ionic states. U_p is the ponderomotive (quiver) energy of a free electron (charge e , mass m_e) in an oscillating laser field of angular frequency ω and electric field amplitude E_0 (Eq. 2)

$$U_p = \frac{e^2 E_0^2}{4m_e \omega^2} \quad (2)$$

As described in detail in the SOM, the Stark-shifted ionization potential $I_p^{(D_j)}$ is the field-free ionization potential plus the difference in Stark shift between the neutral ground state and the j th ionization channel. Due to their differing $I_p^{(D_j)}$, the different ionization channels will have ATI combs shifted with respect to each other. If direct SFI to an electronically excited ion state D_j occurs, the correlated ion fragments will exhibit an ATI comb shifted relative to that correlated with the parent ion. In contrast, if ion excited states were populated subsequently by postionization excitation, meaning that the continuum electron has departed before further excitation occurs, then the fragments would have the same ATI spectrum as the parent ion because postionization excitation can no longer influence the departed continuum electron. Note that whereas several electronically excited ionization channels might correlate with the same ion fragment,

the parent ion will uniquely identify the D_0 ground-state ionization channel. By our method, we can determine whether SFI directly produced ionic ground or excited states, or whether ionic excited states were sequentially populated after removal of the electron. In Table 1 and table S1, we list the vertical and Stark-shifted ionization potentials $I_p^{(D_j)}$ of the lowest four ionic electronic states for the molecules studied here. As seen from Eqs. 1 and 2, the ATI shift depends on laser intensity and, therefore, spatial averaging over the laser focal volume reduces the contrast of the ATI peaks. As discussed in the SOM, we explicitly minimized this effect by using a restricted target volume geometry. We obtained absolute intensity calibrations by studying the shift of the ATI combs with laser intensity (i.e., U_p) for the H_2O molecule, in which the molecular Stark shifts are negligible. In our measurements, the value of U_p in Eq. 1 is very similar for all participating ionic states. This is because, for the low count-rate requirements of our covariance experiment, ionization is spatially restricted to the very center of the laser focus. In SFI, possible excited-state resonances in the neutral molecule can produce the well-known Freeman resonances (35), leading to sharp features in the ATI spectra, which have a limited progression. We indeed observed Freeman resonances in these molecular systems (see fig. S2 and accompanying text), as confirmed by their laser-intensity independence. In contrast, the ATI peaks shift monotonically with laser intensity. In low-frequency fields, the Freeman resonances contribute only to the first two or three ATI peaks: The shifts reported here are based on the higher members of the ATI progression. Therefore, the possible excitation of neutral excited states does not affect our analysis of the ATI comb shifts. By studying CRATI as a function of the ellipticity of the driving field, we furthermore confirmed that electron recollision played no important role in producing excited states.

The details of the experimental geometry, data collection, and analysis are presented in the SOM. In Fig. 1, we present the mass spectrum

(left) and normalized CRATI photoelectron spectra (right) for 1,3-butadiene at a peak intensity of $1.9 \times 10^{13} \text{ W/cm}^2$ ($\hbar\omega = 798 \text{ nm}/1.55 \text{ eV}$, $U_p = 1.16 \text{ eV}$). The mass spectrum is strongly dominated by the parent ion $C_4H_6^+$, which accounts for 87% of the overall ion yield. This number directly gives a lower limit for the probability of formation of the D_0 ionic ground electronic state in SFI of 1,3-butadiene, corresponding to HOMO ionization. A small (13%) contribution from fragments is also seen in the mass spectrum, indicating population of ionic excited states. As a percent ratio of the parent ion signal, the most prominent fragmentation channels are $C_4H_5^+$ (1%), $C_3H_3^+$ (3%), and $C_2H_3^+$ (3%). The CRATI photoelectron spectra (right) show a series of peaks spaced by the photon energy, as expected. The CRATI peaks correlated with the parent $C_4H_6^+$ ion yield a comb associated with ionization into the $X(D_0)$ ground state. In Fig. 1B, the vertical lines indicate the relative shift of the ATI combs, determined from analysis of the higher-order peaks contributing to each comb. Relative to the $C_4H_6^+$ comb, the CRATI combs of the $C_4H_5^+$ and $C_3H_3^+$ fragment channels are both shifted toward lower energy by $0.95 \pm 0.05 \text{ eV}$, as determined by statistical analysis of the relative comb shifts. This is unambiguous experimental evidence of direct SFI into ion excited states in 1,3-butadiene: Postionization fragmentation of the D_0 ground state could not produce this result. From Table 1, the Stark-shifted $D_1(^2A_u)$ and $D_2(^2A_g)$ states of the 1,3-butadiene ion lie 2.3 and 2.5 eV above the Stark-shifted $D_0(^2B_g)$ ground state, respectively. The ATI comb shift can only be observed modulo the photon energy (1.55 eV). Therefore, direct SFI to the $D_1(^2A_u)/D_2(^2A_g)$ states should produce an average CRATI comb shifted relative to that of the ground state by $2.40 - 1.55 = 0.85 \text{ eV}$. Within errors, this is quantitatively the shift observed for the $C_4H_5^+$ and $C_3H_3^+$ CRATI combs. This conclusion is further supported by prior tunable vacuum ultraviolet single-photon ionization studies on 1,3-butadiene, which showed that the $C_4H_5^+$ and $C_3H_3^+$ fragments appear as soon as the $D_1(^2A_u)$ ion state

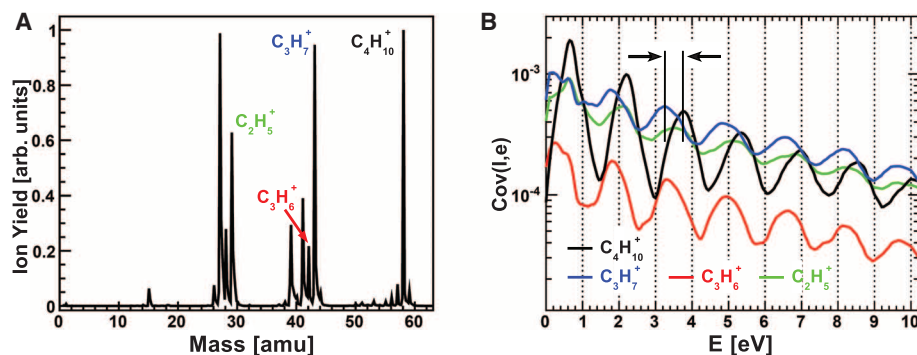


Fig. 2. Strong-field ionization of *n*-butane at a peak intensity of $1.6 \times 10^{13} \text{ W/cm}^2$. (A) Mass spectrum. (B) CRATI photoelectron spectra correlated with different fragment ions. Extensive fragmentation occurs even at low intensity. Several fragment CRATI combs show a shift relative to that of the parent ion (black). These can be quantitatively assigned to originate from direct SFI into excited ionic states.

is energetically accessible (32). In contrast, the $C_2H_3^+$ fragment has an appearance energy more than 6 eV above that of the parent ion (32). In our experiment, $C_2H_3^+$ probably originates from further excitation of the ion in subsequent field cycles. As can be seen in Fig. 1B, the $C_2H_3^+$ CRATI spectrum exhibits fairly washed out ATI structure with little modulation depth, consistent with several SFI channels contributing to the formation of this fragment. We can rule out the population of neutral excited states within the laser pulse, leading to their SFI and associated ATI comb: This is not consistent with the present results because the lower I_P (which determines their ATI comb shift) of a neutral excited state does not match those due to ionization of the neutral ground state.

In Fig. 2, we present mass (left) and CRATI spectra (right) for *n*-butane at a peak intensity of 1.6×10^{13} W/cm² ($\hbar\omega = 798$ nm/1.55 eV, $U_P = 0.93$ eV). In contrast to 1,3-butadiene, *n*-butane predominantly fragments upon ionization, producing $C_3H_n^+$ and $C_2H_n^+$ ions. The $C_3H_7^+$ and $C_3H_6^+$ fragments are associated with populating the first ionic excited state, whereas the $C_2H_5^+$ fragment is known to be associated with the third ionic excited state (33). Relative to the parent ion signal, the percent ratio of the fragment ions are: $C_3H_7^+$ (122%), $C_3H_6^+$ (27%), $C_2H_5^+$ (96%), and $C_2H_3^+$ (152%). Figure 2B shows that the $C_3H_7^+$ fragment CRATI comb (blue) is shifted to lower energy by 0.45 ± 0.05 eV relative to the parent ion comb (black). As seen in Table 1, the energy difference between the Stark-shifted D_0 ion ground state and the field-split $D_1(2^2B_g)/D_2(2^2A_g)$ ionic excited states of *n*-butane is 0.4/0.5 eV, in

quantitative agreement with the shift of the $C_3H_7^+$ comb (blue) in Fig. 2B. This reveals that the dominant $C_3H_7^+$ channel is due to direct SFI into the first excited ionic state. Figure 2B also shows that the $C_3H_6^+$ CRATI comb (red) is shifted to lower energy by 0.40 ± 0.05 eV relative to the parent ion comb. Therefore, within experimental error, we can also associate $C_3H_6^+$ with direct SFI into the first ion excited state. The $C_2H_5^+$ (green) and $C_2H_3^+$ (see SOM) fragment combs are both shifted to lower energy by 0.10 ± 0.05 eV relative to the parent ion comb. Although this shift is not in strict quantitative agreement with the data given in Table 1, within the approximations used in calculating the excited-state polarizabilities, it is consistent with direct SFI into the third ion excited state. The CRATI results for *n*-butane show that SFI to electronically excited continuum channels can dominate over SFI to the ground state. In sum, the combined results of Figs. 1 and 2 experimentally demonstrate that multiple continua do participate in the direct SFI of polyatomic molecules, with the relative contributions of these channels depending on molecular electronic structure. In the following, we interpret these observations in terms of the attosecond molecular SFI response.

The experimental results are independently (that is, using no adjustable parameters) corroborated by all-electron, coupled-channel time-dependent Schrödinger equation (TDSE) calculations, which are outlined in detail in the SOM. Briefly, this mixed orbital/grid-based approach (36) makes no assumptions about the nature of the ionization mechanism or the adiabaticity of the field-driven electron response. The neutral ground state

and the ground and excited ionic states are treated at an appropriate level of ab initio electronic structure theory. Both the bound electrons and the continuum electron are treated in the full three spatial dimensions, whereas the nuclei are kept frozen at the ground-state neutral geometry. The only central approximations are that: (i) a finite number of final ion electronic states are included (seven for 1,3-butadiene, eight for *n*-butane), and (ii) upon excitation, the departing electron is no longer antisymmetrized with respect to the remaining ionic core electrons. Our method supports multiple electronic excitations within the neutral molecule. We calculated the channel-resolved ionization yields to each of the ionic electronic states. To compare with experiment, we additionally averaged these channel-resolved ionization rates over the random lab frame distribution of molecules axes. We calculated the ionization rates using a single half-cycle of the driving laser field, reflecting the subcycle ionization rates and excluding any multicycle response. The use of a half-cycle pulse is discussed and justified in the SOM. In Fig. 3A, we present the results of the calculated channel-resolved SFI yields for the dominant conformers (insets) of 1,3-butadiene (left) and *n*-butane (right) at a peak intensity of 1.5×10^{13} W/cm². For 1,3-butadiene, ionization into the ionic ground state is the dominant channel, with some direct ionization into the first electronically excited state and higher lying channels. This result is in good agreement with the mass spectrum and CRATI results shown in Fig. 1. For the dominant antic conformer of *n*-butane (right), the calculations show that SFI into the first and higher lying electronically excited ionic

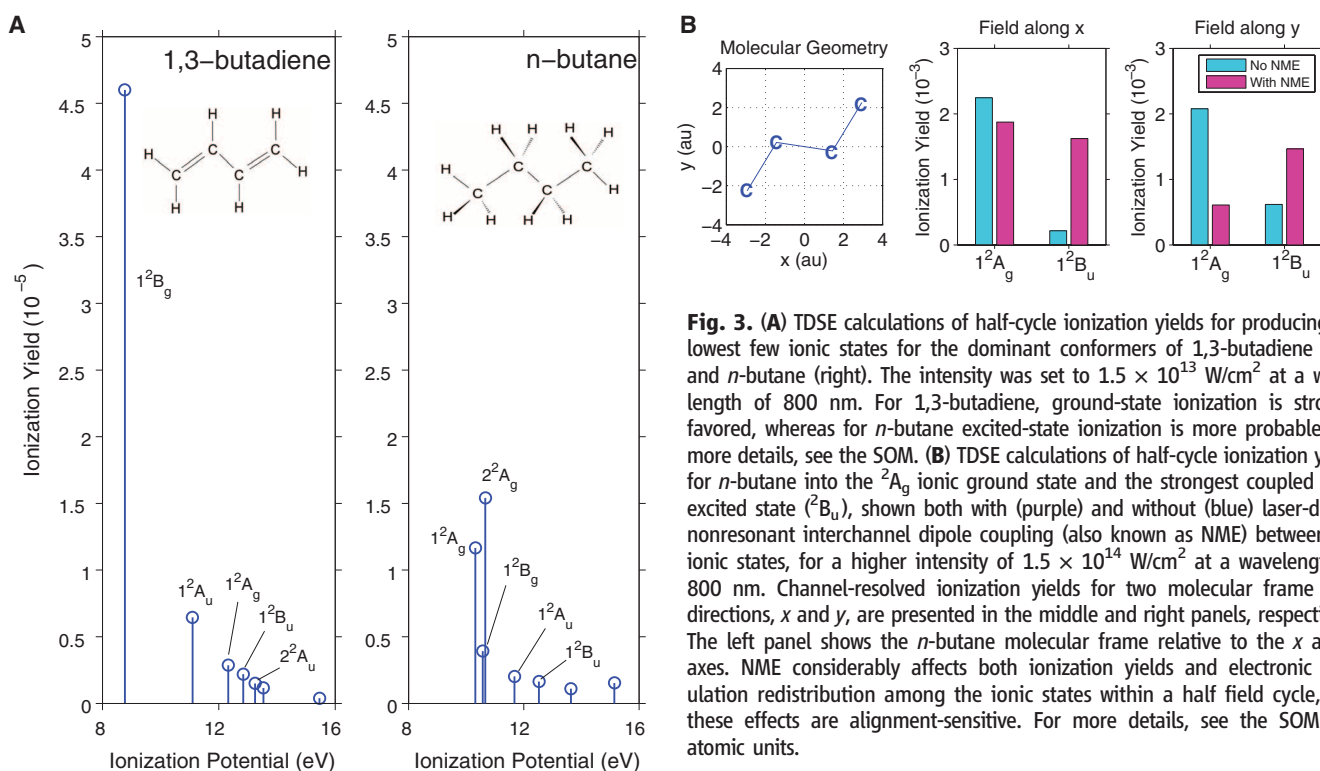


Fig. 3. (A) TDSE calculations of half-cycle ionization yields for producing the lowest few ionic states for the dominant conformers of 1,3-butadiene (left) and *n*-butane (right). The intensity was set to 1.5×10^{13} W/cm² at a wavelength of 800 nm. For 1,3-butadiene, ground-state ionization is strongly favored, whereas for *n*-butane excited-state ionization is more probable. For more details, see the SOM. **(B)** TDSE calculations of half-cycle ionization yields for *n*-butane into the 2^2A_g ionic ground state and the strongest coupled ionic excited state (2^2B_u), shown both with (purple) and without (blue) laser-driven nonresonant interchannel dipole coupling (also known as NME) between the ionic states, for a higher intensity of 1.5×10^{14} W/cm² at a wavelength of 800 nm. Channel-resolved ionization yields for two molecular frame field directions, *x* and *y*, are presented in the middle and right panels, respectively. The left panel shows the *n*-butane molecular frame relative to the *x* and *y* axes. NME considerably affects both ionization yields and electronic population redistribution among the ionic states within a half field cycle, and these effects are alignment-sensitive. For more details, see the SOM. au, atomic units.

states is favored over the ground-state channel. Once again, this agrees well with the experimental mass spectrum and CRATI results for *n*-butane (Fig. 2). These theoretical results show that direct ionization into multiple ionic states can be understood as a subcycle phenomenon. Qualitatively, the SFI response of these two molecules can be understood by considering the ionization energies and Dyson orbitals given in table S4. The Dyson orbitals represent the initial single-particle state of the ionizing electron. Within the Hartree-Fock approximation and Koopmans picture, they become ionization channels from the HOMO, HOMO–1, HOMO–2, etc. In a tunneling picture, SFI depends exponentially on the I_p through the Keldysh factor $\exp[-(2/3)(2I_p)^{3/2}/E_0]$. Furthermore, for diatomics (37) it is known that nodal planes in the Dyson orbitals can cause strong suppression of the total yield of SFI. For the case of 1,3-butadiene, although the 1^2A_u excited state has fewer nodal planes than the 1^2B_g ground state, the large energy gap favors the ground state. For the case of *n*-butane, where the energy gaps are much smaller, orbital symmetry effects become more pronounced. The Dyson orbitals for *n*-butane reveal that the 2^2A_g excited state has the fewest nodal planes; hence, this state experiences the least suppression, rendering it the dominant SFI channel as seen in Fig. 3A.

An important question is the role of laser-driven nonadiabatic interchannel coupling (also known as NME) in polyatomics. As discussed in the SOM, the hydrocarbons irradiated here at a peak intensity of $\sim 10^{13}$ W/cm² (798 nm) exhibited predominantly adiabatic multichannel SFI dynamics. However, at higher intensities, the role of NME in even simple diatomics remains under active debate (6, 15). With our TDSE method, we can artificially turn off the laser-driven interchannel coupling in the theory and compare the result to the case where the coupling is retained. This procedure accentuates the effects of NME on the ionic-state population distribution during SFI. In Fig. 3B, we present theoretical channel-resolved SFI yields in *n*-butane using a half-cycle pulse of higher intensity (1.5×10^{14} W/cm², 800 nm), both with (purple) and without (blue) NME coupling. For simplicity, only the 2^2A_g ground state and the 2^2B_u excited state were included, as these are the continuum channels most strongly dipole-coupled by the field. As seen in Fig. 3B, along two molecular frame directions *x* (middle) and *y* (right), coherent subcycle redistribution of population between electronic continuum channels occurs when NME is included. This qualitatively demonstrates that, at intensities typical of HHG experiments, the nonadiabatic population of multiple electronic continua can be another important contributor to the attosecond molecular response.

Although the present study does not address the tacit coherences created between ionic states (2), the CRATI method directly reveals the population of multiple electronic continua

in the SFI of polyatomic molecules. A future extension of this method that measures fragment ion energy and/or angular distributions will be of interest. Studying CRATI spectra as a function of molecular electronic structure, molecular frame alignment, and laser frequency/intensity will illuminate the role of multiple electronic continua in attosecond SFI spectroscopies such as HHG.

References and Notes

1. F. Krausz, M. Yu. Ivanov, *Rev. Mod. Phys.* **81**, 163 (2009).
2. E. Goulielmakis *et al.*, *Nature* **466**, 739 (2010).
3. G. Sansone *et al.*, *Nature* **465**, 763 (2010).
4. M. Uiberacker *et al.*, *Nature* **446**, 627 (2007).
5. O. Smirnova *et al.*, *Nature* **460**, 972 (2009).
6. S. Haessler *et al.*, *Nat. Phys.* **6**, 200 (2010).
7. H. J. Wörner, J. B. Bertrand, D. V. Kartashov, P. B. Corkum, D. M. Villeneuve, *Nature* **466**, 604 (2010).
8. J. Itatani *et al.*, *Nature* **432**, 867 (2004).
9. C. Vozzi *et al.*, *Appl. Phys. Lett.* **97**, 241103 (2010).
10. Y. Huismans *et al.*, *Science* **331**, 61 (2011).
11. M. Meckel *et al.*, *Science* **320**, 1478 (2008).
12. P. B. Corkum, *Phys. Rev. Lett.* **71**, 1994 (1993).
13. X.-B. Zhou *et al.*, *Phys. Rev. Lett.* **102**, 073902 (2009).
14. H. Akagi *et al.*, *Science* **325**, 1364 (2009).
15. S. Petretti, Y. V. Vanne, A. Saenz, A. Castro, P. Decleva, *Phys. Rev. Lett.* **104**, 223001 (2010).
16. M. Lezius *et al.*, *Phys. Rev. Lett.* **86**, 51 (2001).
17. M. Smits, C. A. de Lange, A. Stolow, D. M. Rayner, *Phys. Rev. Lett.* **93**, 203402 (2004).
18. A. N. Markevitch *et al.*, *Phys. Rev. A* **68**, 011402 (2003).
19. Z. B. Walters, S. Tonzani, C. H. Greene, *J. Phys. Chem. A* **112**, 9439 (2008).
20. D. J. Fraser *et al.*, *J. Phys. B* **28**, L739 (1995).
21. P. Agostini, F. Fabre, G. Mainfray, G. Petite, N. Rahman, *Phys. Rev. Lett.* **42**, 1127 (1979).
22. K. J. Schafer, B. Yang, L. F. DiMauro, K. C. Kulander, *Phys. Rev. Lett.* **70**, 1599 (1993).
23. C. I. Blaga *et al.*, *Nat. Phys.* **5**, 335 (2009).
24. H. Rottke, J. Ludwig, W. Sandner, *J. Phys. At. Mol. Opt. Phys.* **29**, 1479 (1996).
25. X. Zhou *et al.*, *Phys. Rev. Lett.* **100**, 073902 (2008).
26. B. K. McFarland, J. P. Farrell, P. H. Bucksbaum, M. Gühr, *Science* **322**, 1232 (2008).
27. I. Thomann *et al.*, *J. Phys. Chem. A* **112**, 9382 (2008).
28. W. Li *et al.*, *Science* **322**, 1207 (2008).
29. P. B. Armentrout, T. Baer, *J. Phys. Chem.* **100**, 12866 (1996).
30. W. A. Chupka, in *Chemical Spectroscopy and Photochemistry in the VUV*, C. Sandorfy, P. Ausloos, M. B. Robin, Eds. (North Atlantic Treaty Organization Advanced Study Institute Series, Reidel, Dordrecht, 1974), pp. 433–464.
31. R. Bombach, J. Dannacher, J.-P. Stadelmann, *J. Am. Chem. Soc.* **105**, 1824 (1983).
32. J. Dannacher, J.-P. Flamme, J.-P. Stadelmann, J. Vogt, *Chem. Phys.* **51**, 189 (1980).
33. W. A. Chupka, J. Berkowitz, *J. Chem. Phys.* **47**, 2921 (1967).
34. L. J. Frasinski *et al.*, *Phys. Rev. A* **46**, R6789 (1992).
35. R. R. Freeman *et al.*, *Phys. Rev. Lett.* **59**, 1092 (1987).
36. M. Spanner, S. Patchkovskii, *Phys. Rev. A* **80**, 063411 (2009).
37. J. Muth-Böhm, A. Becker, F. H. M. Faisal, *Phys. Rev. Lett.* **85**, 2280 (2000).
38. G. Bieri, F. Burger, E. Heilbronner, J. P. Maier, *Chim. Acta* **60**, 2213 (1977).
39. D. M. P. Holland *et al.*, *J. Phys. At. Mol. Opt. Phys.* **29**, 3091 (1996).

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The Role of Driving Energy and Delocalized States for Charge Separation in Organic Semiconductors

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The electron-hole pair created via photon absorption in organic photoconversion systems must overcome the Coulomb attraction to achieve long-range charge separation. We show that this process is facilitated through the formation of excited, delocalized band states. In our experiments on organic photovoltaic cells, these states were accessed for a short time (<1 picosecond) via infrared (IR) optical excitation of electron-hole pairs bound at the heterojunction. Atomistic modeling showed that the IR photons promote bound charge pairs to delocalized band states, similar to those formed just after singlet exciton dissociation, which indicates that such states act as the gateway for charge separation. Our results suggest that charge separation in efficient organic photoconversion systems occurs through hot-state charge delocalization rather than energy-gradient-driven intermolecular hopping.

In contrast to the elegant photosynthetic apparatus evolved by nature (1), organic photovoltaic (OPV) cells use a single heterojunction between two semiconductors to generate charge (2). These semiconductors, referred to as the do-

nor (D) and acceptor (A), are cast from solution or vacuum sublimed to form a thin film with nanoscale domains of relatively pure “bulk” materials and large interfacial regions. This architecture, known as the bulk heterojunction (3),

allows for absorption of light in the bulk domains to form excitons followed by charge generation at the interface where offsets between the lowest unoccupied molecular orbitals (LUMOs) and highest occupied molecular orbitals (HOMOs) of the D and A enable electron transfer, thereby dissociating the exciton.

The various conjugated macromolecules used in this study are shown in Fig. 1, along with their reported external quantum efficiencies (EQEs), under short-circuit conditions, which vary from high (>70%) to low (<10%) (4–9). This variation in performance cannot be explained simply by energy-level offsets, as these are sufficient in all listed systems to enable exciton dissociation, nor can they be explained by nonideal charge transport (10, 11). Instead, fundamental differences in electronic structure of the heterojunctions formed between materials affect the efficiency of photoconversion.

The current understanding of the photon-to-charge conversion in OPV devices is illustrated in Fig. 2, A to C (12). Absorption of a photon produces an intramolecular singlet exciton at the D-A interface either directly or after exciton diffusion from the bulk domains. The exciton then undergoes ultrafast quasi-adiabatic charge transfer, yielding the hole on D and electron on A. Having overcome the intramolecular Coulomb attraction (holding together the singlet exciton), via the use of offset energy levels, the charges remain electrostatically attracted across the D-A interface (10, 13). Because of the interaction, the nature of the state produced may differ from that of a free well-separated electron-hole pair (separated charges, SC), so hereafter we refer to the species formed immediately after charge transfer as “charge-transfer” (CT) states, without consideration of whether these states eventually dissociate into free carriers or stay bound until recombination (14). When electron and hole do localize, so that they are immediately adjacent on either side on the heterojunction, Coulombic binding energies of several hundred meV are expected and observed (15, 16). In photosynthesis, separation of charges is achieved with the aid of cascaded energy levels and ion screening (1, 17). OPVs, by contrast, possess none of these elements, yet surprisingly, some OPV systems work very efficiently (Fig. 1). Hence, the fundamental question of how the organic heterojunction enables efficient long-range charge separation remains unanswered.

Here, we address this issue by applying an electro-optical pump-push experiment, performed

on working OPV diodes (Fig. 2D). A visible “pump” pulse illuminates the OPV and creates a population of CT and SC states, as illustrated in Fig. 2, A and C. The pump flux is kept low to give excitation densities $\sim 10^{16} \text{ cm}^{-3}$, comparable to continuous solar excitation (18). The charge carrier on the polymer causes a geometrical rearrangement of the chain, known as polaron formation. Polaron formation lowers the energy for charge storage by pulling two band states (π and

π^*) into the gap, creating the localized polaron illustrated in Fig. 2B (19, 20). For the hole polaron, the lower gap state is singly occupied, and the optical transition (P1) between it and the π valence band is strongly allowed, forming an absorption band typically between 0.1 and 0.5 eV (20, 21), with the energy being determined by the degree of polaron delocalization (22). As we show below, polaron formation plays a key role in limiting long-range charge separation. In

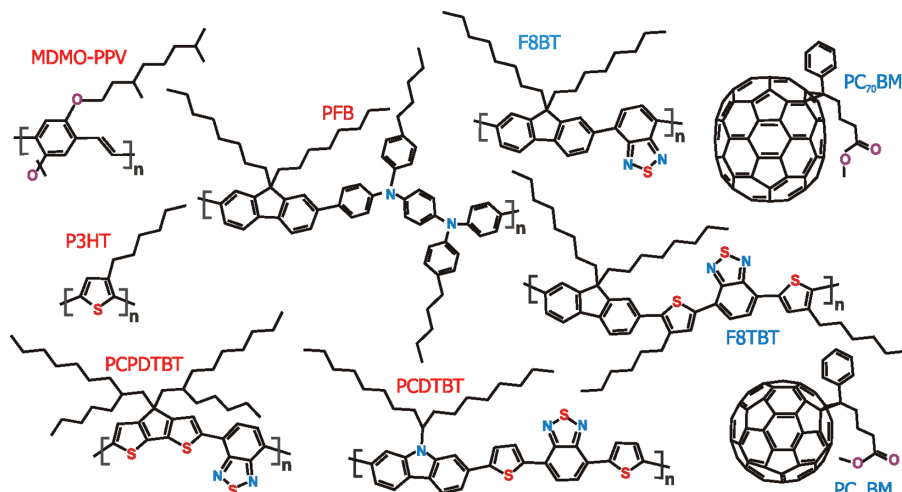


Fig. 1. Chemical structures of donor (left, red captions) and acceptor (right, blue captions) materials used in this study. Typical external quantum efficiencies of the corresponding OPVs (4–9), defined as the number of collected electrons collected per incident photon, are as follows: PFB:F8BT (6%), P3HT:F8TBT (25%), MDMO-PPV:PC₇₀BM (40%), PCPDTBT:PC₇₀BM (50%), P3HT:PC₆₀BM (70%), and PCDTBT:PC₇₀BM (80%).

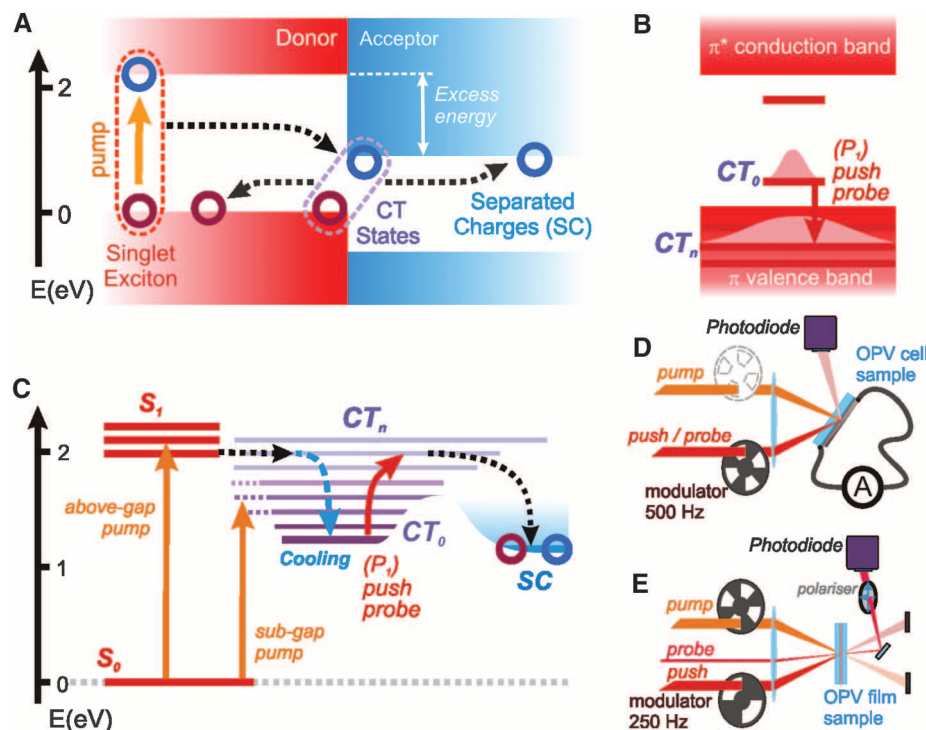


Fig. 2. (A and B) Band diagrams for (A) a typical OPV and (B) a cationic state on the polymer donor. (C) Free-energy state diagram of the same OPV system. Singlet, charge-transfer (CT; lowest-lying, CT₀) band states, CT_n, and separated-charges (SC) states are shown; positive charge density distribution in (B) is indicated by pink contour. Solid arrows show optical transitions, and dashed arrows indicate energy- and charge-transfer pathways involved in photoconversion. Layouts of (D) pump-push photocurrent and (E) three-pulse transient-anisotropy experiment.

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our experiments, we address the dynamics of electron-hole pairs still localized at the interface. After charge transfer, the CT_n states formed are “hot” states with energies of 0.3 to 1 eV in excess of the lowest-lying CT_0 states. The charge pair then relaxes through the manifold to the lowest-

lying CT_0 states, giving rise to a red-shifted CT emission (23). Here we selectively excited the $P1$ (CT_0 to CT_n) transition with an infrared (IR) “push” pulse, repopulating hot states that the system samples immediately after the charge transfer. The perturbation of the charge dynamics on

the molecular level affected the OPV performance, and the corresponding change of the photocurrent (δPC) induced by the IR push pulse is recorded as a function of the delay between pump and push (24, 25). In a complementary all-optical experiment (Fig. 2E), the effect of the push pulse

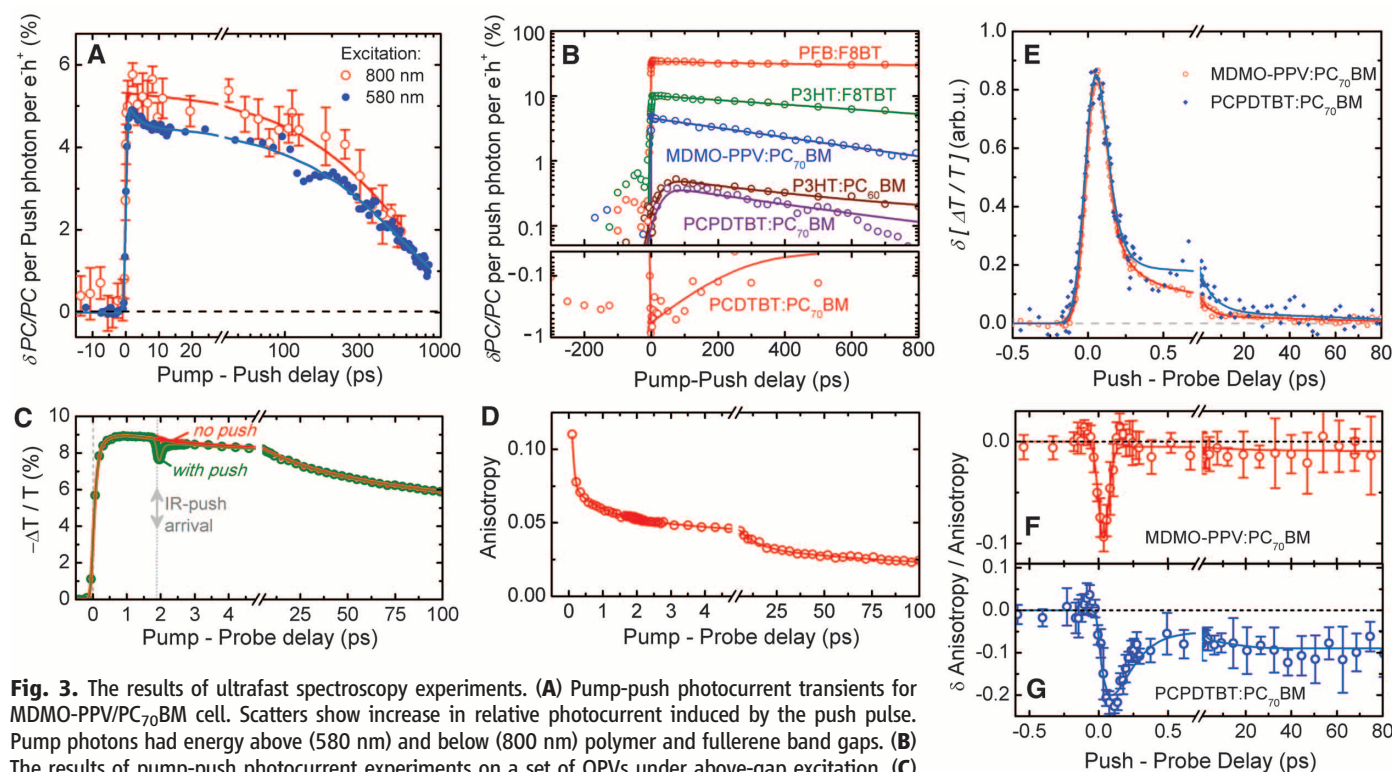


Fig. 3. The results of ultrafast spectroscopy experiments. (A) Pump-push photocurrent transients for MDMO-PPV/PC₇₀BM cell. Scatters show increase in relative photocurrent induced by the push pulse. Pump photons had energy above (580 nm) and below (800 nm) polymer and fullerene band gaps. (B) The results of pump-push photocurrent experiments on a set of OPVs under above-gap excitation. (C) Transient-absorption and (D) transient-anisotropy kinetics for MDMO-PPV:PC₇₀BM film excited at 580 nm and probed at 3 μ m. Green line in (C) shows dynamics with push pulse arriving at ~ 2 ps delay. The effect of push pulse on the isotropic (E) and anisotropic (F and G) transients, calculated from the difference between with-push and no-push measurements, for MDMO-PPV/PC₇₀BM, PCPDTBT/PC₇₀BM films. In all figures, solid lines are (multi)exponential fits convoluted with the Gaussian function at zero delay.

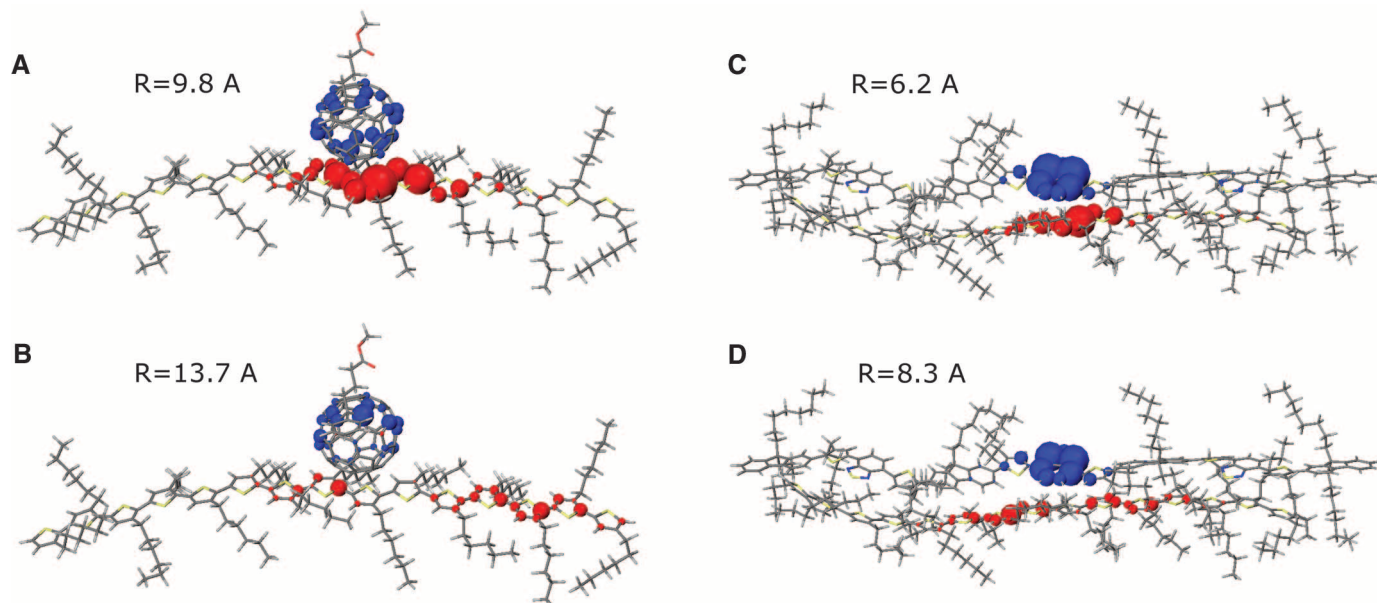


Fig. 4. Microelectrostatic simulations of the charge distribution at the P3HT/PCBM (A and B) and P3HT/F8TBT (C and D) heterojunction, with electron and hole densities shown in blue and red, respectively. (A and C) Charge dis-

tribution in the lowest CT -state configuration, and (B and D) in the excited CT -state configuration created upon absorption of an IR-push photon. R , average electron-hole separation.

on charge dynamics is observed with an additional IR “probe” pulse (26). To boost the signal and ensure that a large number of charges were excited by the push pulse, we used a higher excitation density ($\sim 10^{19} \text{ cm}^{-3}$) in the all-optical experiment.

The push-induced states were similar to those populated directly after exciton dissociation. The geometry of the polymer was different in the case of the push experiment because the chain had relaxed to form a polaronic state. However, as established earlier (19), the presence of the polaron distortion leaves all of the other π band states unchanged, as is also confirmed by the calculation presented in fig. S3 (27). This allowed us to generalize the results of our experiments to photoconversion under solar-illumination conditions.

Figure 3A shows pump-push photocurrent data for an MDMO-PPV/PC₇₀BM solar cell. When the push pulse arrived after the pump, the photocurrent manifestly increased. The rise time of this increase was instrument-limited (200 fs), indicating that the push interacted with species created directly after excitation. The magnitude of the push-induced photocurrent decayed slowly (~ 600 ps) as the delay time increased. We attribute this decay to the CT-state lifetime, in agreement with previous time-resolved photoluminescence measurements (28, 29). From measurements of the pump-induced absorption at the push wavelength and the flux of the push pulse, we calculated the increase in the photocurrent per IR photon absorbed per charge pair. For an MDMO-PPV/PC₇₀BM cell, we observed a $\sim 5\%$ change in relative photocurrent i.e., every IR photon absorbed by an electron-hole pair had a 5% chance of helping to dissociate it. The fast rise time (200 fs) ruled out charge trapping after diffusion to possible defect sites. We observed a longer rise of the signal for blends of P3HT and PCPDTBT, which was probably associated with delayed formation of bound states preceded by the exciton diffusion.

An important question with regard to charge separation is the role of “excess energy” that the hot CT_n state inherited from the exciton. It has been argued that this energy, which is lost during relaxation to the “cold” CT₀ state, facilitates long-range charge separation (11, 30). However, this interpretation has been questioned (31), and the role of excess energy remains ambiguous (14, 32–35). We elucidated the effect of excess energy on charge separation by using below-gap excitation (Fig. 3A, open circles) (31, 36). Through the below-gap transition, CT states were populated directly at the D-A interface, rather than through singlet exciton dissociation. Although the cross-section of the $S_0 \rightarrow \text{CT}$ transition was low, it allowed for charge generation while bypassing the hot CT state. We note that this excitation was not necessarily to the cold CT state, but occurs with ground-state D and A geometries. Subsequent geometrical relaxation formed polaron states, giving Stokes-shifted (~ 0.6 eV) CT luminescence (37). Although the CT state excited had ~ 0.5 eV less “excess energy” than the state created after exci-

ton dissociation, $\delta\text{PC}/\text{PC}$ kinetics (Fig. 3A) were similar for sub- and above-gap excitations, demonstrating that charge separation effectively occurred without the need for the large excess energy associated with the exciton (31).

Figure 3B shows pump-push transients corresponding to above-gap excitation for a range of materials systems. The relative amount of additional photocurrent stimulated by re-excitation was very dependent on the particular material. Polymer-polymer blends, PFB/F8BT and P3HT/F8BT, displayed a large push-induced increase of photocurrent (10 to 50%). Consistent with previous reports, the CT-state lifetime in these materials was up to a few tens of nanoseconds (38). Polymer-fullerene cells demonstrated a modest photocurrent increase ($<6\%$) and subnanosecond CT-state lifetimes. Finally, the highly efficient PCDTBT/PC₇₀BM system showed a minor decrease in photocurrent upon IR irradiation, possibly the result of bimolecular effects.

Both free hole polarons (SC) and those bound within CT states may absorb the IR push pulse. Although recent studies have indicated that the photoinduced absorption at 0.5 eV is related to CT states (39, 40), IR push photons can be also absorbed by weakly bound charges, which would contribute to the PC even without the application of the push pulse. Thus, systems with lower EQE, where the amount of bound charges is high, demonstrate greater $\delta\text{PC}/\text{PC}$ response. By contrast, in the efficient photoconversion systems, where the number of CT states is reduced, the push-induced photocurrent is low. After normalization on the estimated amount of bound charges, we found that for all materials, the efficiency of charge separation from the CT state after the push was lower (roughly 50%) but still comparable to the efficiency of charge separation after the original pump pulse. This result indicates that the push pulse took those excitations that had relaxed to bound CT states back to states similar to the early-time hot states formed after the singlet exciton dissociation, giving them a second chance to dissociate.

To monitor the effect of the push pulse on the charges directly, we used an all-optical pump-push-probe technique (Fig. 2E). Figure 4A shows isotropic pump-probe transients in a MDMO-PPV/PC₇₀BM film with and without the push pulse. Pump pulses created CT and SC states in the OPV material, and their evolution was monitored with the IR probe pulse. When the probe arrived after the pump, it was partly absorbed by the pump-generated carriers. The red curve in Fig. 3C reflects the population of all charged (CT₀ and SC) states as a function of time. The green circles present analogous dynamics when a push pulse, ~ 2 ps after the pump, brought a substantial fraction ($\sim 10\%$) of the charges back to the hot state CT_n (Fig. 2, B and C). This re-excitation was observed as a sudden drop in signal amplitude because fewer charges were available to absorb the probe beam. The signal then rejoined the red curve as the CT_n states relaxed to CT₀. Figure

3E presents the difference in response with and without the push pulse, for MDMO-PPV and PCPDTBT mixed with PC₇₀BM. A fast sub-100 fs component accounted for $\sim 80\%$ of the signal decay, with the residual relaxation of $\sim 20\%$ of states occurring on a longer but still relatively fast 1- to 20-ps time scale.

Figure 3D presents the anisotropic component of the same pump-probe transient in an MDMO-PPV/PC₇₀BM film. The initial anisotropy was lower than the 0.4 limit for random distribution of dipoles, indicating that there may be (excitonic) dynamics occurring before charge generation and/or below our ~ 70 -fs time resolution (34). However, the nonzero anisotropy observed indicates that the P1 transition dipole of charges partly inherited polarization memory from the singlet excitons. As charges moved from the chain on which they were created to another chain, the transition dipole moment associated with the charge acquired a different orientation, which was observed as depolarization (34).

Figure 3, F and G, shows the relative difference between “with-push” and “no-push” anisotropic transients. As a general trend, we observed that anisotropy was reduced by the push. The anisotropy within the pulse-overlap region dropped, probably because of the different orientation of the P1 transition dipoles in the excited state. Anisotropy dynamics at longer (>0.2 ps) delays displayed biphasic behavior. Anisotropy was lost after the push and decreased further in the following 10 to 50 ps. The loss in anisotropy after the push corresponded to the re-excited hot CT state relocalizing to the neighboring CT/SC state with a different orientation of charge-associated dipole (13). The continued loss of anisotropy provides further evidence that charges became more mobile after the push pulse.

The fast charge relaxation and relocalization observed in three-pulse experiments are not consistent with the currently dominant models of charge separation through relaxation-assisted intermolecular hopping. However, the observed results can be explained by hole delocalization achieved via band states. These states are formed after singlet exciton dissociation, before polaron formation occurs. Here we repopulate these states through the optically allowed IR transition. The absorption of the IR photon promotes an electron from the delocalized valence band to the localized HOMO, instantaneously delocalizing the hole, as illustrated schematically in Fig. 2B. Such delocalization would then play a critical role in overriding the Coulomb binding energy (41).

To confirm this hypothesis, we resorted to atomistic “many-body” modeling. We first demonstrated that optical excitation from the singly (positively) charged ground state to the lowest dipole-allowed electronic state, namely, the CT₀ $\rightarrow$ CT_n (P1) electronic transition in the simplified one-electron picture above, resulted in an increased intrachain hole delocalization in isolated chains of archetypal conjugated polymers (27). By combining force-field geometry optimization with

quantum-chemical excited-state calculations of D-A pairs, we then inferred the nature of the electronic states at representative polymer-PCBM and polymer-polymer heterojunctions. Figure 4A features the charge density distribution as calculated in the lowest charge-transfer electronic excited state (CT_0) of a P3HT/PCBM heterojunction, and Fig. 4B shows the analogous situation for a higher-lying, strongly dipole coupled to CT_0 , excited state, CT_n , prepared by absorption of an IR photon from CT_0 . The P3HT-hole wave function, which was confined close to the PCBM electron in the CT_0 state, was more delocalized along the polymer backbone in the CT_n state, resulting in an increased average intermolecular electron-hole separation. The electron density on the fullerene was also changed upon the excitation to the CT_n state; however, as the electron is already delocalized on the fullerene molecule, this effect is not very pronounced. Nonetheless, current calculations cannot exclude further delocalization over more than one fullerene molecule when such molecules are available. Similar results were obtained for the P3HT/F8TBT interface (Fig. 4, C and D). Thus, at both heterojunctions, the strong IR transition from the CT_0 to the CT_n excited state, which correlates with the valence $\rightarrow n$ transition in a single P3HT chain, delocalizes the hole wave function along the donor chain and thereby promotes a larger intermolecular electron-hole separation. Although this transient delocalization is short-lived (subpicosecond), as measured by the all-optical technique in Fig. 4, B and D, it allows for charges to decouple and move apart at longer time scales (10 to 50 ps).

Although the general trend of delocalization of charge by optical excitation is observed for both modeled systems, the extent of delocalization is material dependent. Moving from F8TBT to PCBM increases the average electron-hole separation by 50%. This, in turn, induces even larger variations in CT-state binding energy and dissociation probability. The results of these variations are clearly seen in Figs. 1 and 3B. This difference in delocalization of charges causes OPV systems to demonstrate markedly different quantum efficiencies.

We propose that the driving energy for charge separation in organic photoconversion systems is the energy needed to reach delocalized band states, which are critical for long-range charge separation. Although the delocalized states are extremely short-lived (<1 ps), they enable charges to override the otherwise dominant Coulomb interaction. By contrast, large band offsets are not crucial for free-charge formation. Our results provide a new framework to understand charge generation in organic systems and outline the basis for the design of improved OPVs. In particular, those materials that support delocalized charge wave functions and have low reorganization energies due to structural rigidity and suppressed torsion relaxation should be targeted for the next generation of OPVs. This approach would mitigate the problem of polaron formation and allow

for efficient charge separation with minimal band offsets, greatly increasing the open-circuit voltage and efficiency of OPVs. The superior performance of fullerene-based OPVs is explained by meeting the outlined criteria, as is the performance of recently reported high-efficiency OPVs based on porphyrins (42, 43).

References and Notes

- R. E. Blankenship, *Molecular Mechanisms of Photosynthesis* (Blackwell Science, Oxford, 2002).
- T. M. Clarke, J. R. Durrant, *Chem. Rev.* **110**, 6736 (2010).
- G. Yu, J. Gao, J. C. Hummelen, F. Wudl, A. J. Heeger, *Science* **270**, 1789 (1995).
- M. M. Wienk et al., *Angew. Chem. Int. Ed.* **42**, 3371 (2003).
- Y. Kim et al., *Nat. Mater.* **5**, 197 (2006).
- J. Peet et al., *Nat. Mater.* **6**, 497 (2007).
- C. R. McNeill et al., *Adv. Funct. Mater.* **18**, 2309 (2008).
- R.-Q. Peng et al., *Nat. Mater.* **9**, 152 (2010).
- S. H. Park et al., *Nat. Photonics* **3**, 297 (2009).
- C. Deibel, T. Strobel, V. Dyakonov, *Adv. Mater. (Deerfield Beach Fla.)* **22**, 4097 (2010).
- S. Shoaee et al., *J. Am. Chem. Soc.* **132**, 12919 (2010).
- J.-L. Brédas, J. E. Norton, J. Cornil, V. Coropceanu, *Acc. Chem. Res.* **42**, 1691 (2009).
- T. Strobel, C. Deibel, V. Dyakonov, *Phys. Rev. Lett.* **105**, 26602 (2010).
- F. Etzold et al., *J. Am. Chem. Soc.* **133**, 9469 (2011).
- S. Gélinas et al., *J. Phys. Chem. C* **115**, 7114 (2011).
- A. C. Morteani, P. Sreearunothai, L. M. Herz, R. H. Friend, C. Silva, *Phys. Rev. Lett.* **92**, 247402 (2004).
- M. R. Jones, *Biochem. Soc. Trans.* **37**, 400 (2009).
- J. Piris et al., *J. Phys. Chem. C* **113**, 14500 (2009).
- S. A. Brazovskii, N. N. Kirova, *JETP Lett.* **33**, 4 (1981).
- D. K. Campbell, A. R. Bishop, K. Fesser, *Phys. Rev. B* **26**, 6862 (1982).
- X. Wei, Z. V. Vardeny, N. S. Sariciftci, A. J. Heeger, *Phys. Rev. B* **53**, 2187 (1996).
- R. Österbacka, C. P. An, X. M. Jiang, Z. V. Vardeny, *Science* **287**, 839 (2000).
- K. Vandewal, K. Tvingstedt, A. Gadisa, O. Inganäs, J. V. Manca, *Nat. Mater.* **8**, 904 (2009).
- J. G. Müller et al., *Phys. Rev. B* **72**, 195208 (2005).
- J. G. Müller, U. Lemmer, J. Feldmann, U. Scherf, *Phys. Rev. Lett.* **88**, 147401 (2002).
- S. V. Frolov, Z. Bao, M. Wohlgenannt, Z. V. Vardeny, *Phys. Rev. B* **65**, 205209 (2002).
- Materials and methods are available as supporting material on Science Online.
- M. A. Loi et al., *Adv. Funct. Mater.* **17**, 2111 (2007).
- D. Veldman et al., *J. Am. Chem. Soc.* **130**, 7721 (2008).
- R. D. Pensack, J. B. Asbury, *J. Am. Chem. Soc.* **131**, 15986 (2009).
- J. Lee et al., *J. Am. Chem. Soc.* **132**, 11878 (2010).
- V. I. Arkhipov, P. Heremans, H. Bassler, *Appl. Phys. Lett.* **82**, 4605 (2003).
- D. Veldman, S. C. J. Meskers, R. A. J. Janssen, *Adv. Funct. Mater.* **19**, 1939 (2009).
- A. A. Bakulin, D. Martynov, D. Y. Paraschuk, P. H. M. van Loosdrecht, M. S. Pshenichnikov, *Chem. Phys. Lett.* **482**, 99 (2009).
- R. A. Marsh, J. M. Hodgkiss, R. H. Friend, *Adv. Mater. (Deerfield Beach Fla.)* **22**, 3672 (2010).
- J. J. Benson-Smith et al., *Adv. Funct. Mater.* **17**, 451 (2007).
- K. Vandewal, K. Tvingstedt, A. Gadisa, O. Inganäs, J. V. Manca, *Phys. Rev. B* **81**, 125204 (2010).
- Y. S. Huang et al., *Nat. Mater.* **7**, 483 (2008).
- J. Holt, S. Singh, T. Drori, Y. Zhang, Z. V. Vardeny, *Phys. Rev. B* **79**, 195210 (2009).
- T. Drori, J. Holt, Z. V. Vardeny, *Phys. Rev. B* **82**, 075207 (2010).
- G. D. Scholes, *ACS Nano* **2**, 523 (2008).
- T. Matsuo et al., *J. Am. Chem. Soc.* **131**, 16048 (2009).
- R. F. Service, *Science* **332**, 293 (2011).

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Supporting Online Material

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Climatic Niche Shifts Are Rare Among Terrestrial Plant Invaders

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The assumption that climatic niche requirements of invasive species are conserved between their native and invaded ranges is key to predicting the risk of invasion. However, this assumption has been challenged recently by evidence of niche shifts in some species. Here, we report the first large-scale test of niche conservatism for 50 terrestrial plant invaders between Eurasia, North America, and Australia. We show that when analog climates are compared between regions, fewer than 15% of species have more than 10% of their invaded distribution outside their native climatic niche. These findings reveal that substantial niche shifts are rare in terrestrial plant invaders, providing support for an appropriate use of ecological niche models for the prediction of both biological invasions and responses to climate change.

Niche conservatism in space and time is a key assumption for predicting the impact of global change on biodiversity (1, 2). It is particularly important for the anticipation of biological invasions, which can cause severe dam-

age to biodiversity, economies, and human health (3). Niche conservatism implies that species tend to grow and survive under the same environmental conditions in native and invaded ranges (2). However, the generality of this assumption is

challenged by recent evidence suggesting that the climatic niche occupied by species may not be conserved between their native and invaded ranges, as documented by observed niche shifts for plants (4, 5), insects (6, 7), and fish (8). Yet, several of these studies have focused on a single species [e.g., (4, 6, 7)] or have used controversial niche overlap metrics [e.g., (5, 8); based on 26 and 18 species, respectively], making it difficult to assess the generality of this phenomenon among alien invasive species. Therefore, the question of whether niche shifts represent a prominent or unusual phenomenon among alien invasive species remains largely unresolved (9).

There are two major reasons why niche conservatism during biological invasion needs further investigation. First, anticipation is the most effective management strategy (10), and niche conservatism is a strong and necessary assumption to predict invasion risk for specific regions (1, 2). Ecological niche models (ENMs) (11, 12), the most commonly used predictive tool in this regard, are traditionally calibrated using native species distributions and then projected onto other continents to highlight areas susceptible to invasions (13). Second, detecting significant deviations from niche conservatism may highlight invasive species that are characterized by ecological (14, 15) or evolutionary changes (16, 17) during invasions, helping us understand when such changes are likely to occur, which is crucial in an era of rapid climate change.

When the niche of a species changes, its mean position (centroid) is likely to move within a multivariate environmental niche space. However, describing the shift of the centroid (4, 5, 7) falls short in helping to understand processes affecting the niche, because niche changes can affect both the position and shape of a niche. This is, for example, the case when species expand to new climates at one particular niche margin (1, 4) and only partially fill the niche (i.e., unfilling) at another (18) (e.g., due to dispersal limitation) (fig. S1). Assuming a species is at equilibrium in its native range (that is, it has colonized all suitable environments), expansion to climates that are new to the species but available in the native range should be considered unambiguously as niche shifts (fig. S1) (12); i.e., resulting from changes in biotic interactions or rapid evolution of the species (1). This conceptual approach to detecting niche shifts is important because analyses of empirical field data alone cannot determine whether the expansion to climates not available in the native range (that is, nonanalog climates) represents a true niche shift or the filling of a

preadapted niche. On the other hand, unfilling in the invaded range is more likely due to dispersal limitation, because biological invasions are recent and ongoing phenomena.

Niche changes due to unfilling have been considered niche shifts in previous studies (4–7), but our analyses (12) reveal that many of these reflect ongoing colonization instead, indicating that the species is likely to invade additional geographic

regions in the future (13). Thus, metrics of niche shift are very sensitive to the underlying statistical and conceptual assumptions, and a solid conceptual foundation for identifying ecologically meaningful and statistically significant niche changes has only recently been developed (12, 19–21).

Here, we disentangle and quantify the amount of niche overlap, niche expansion, and niche unfilling (see figs. S1 and S2) for 50 Holarctic

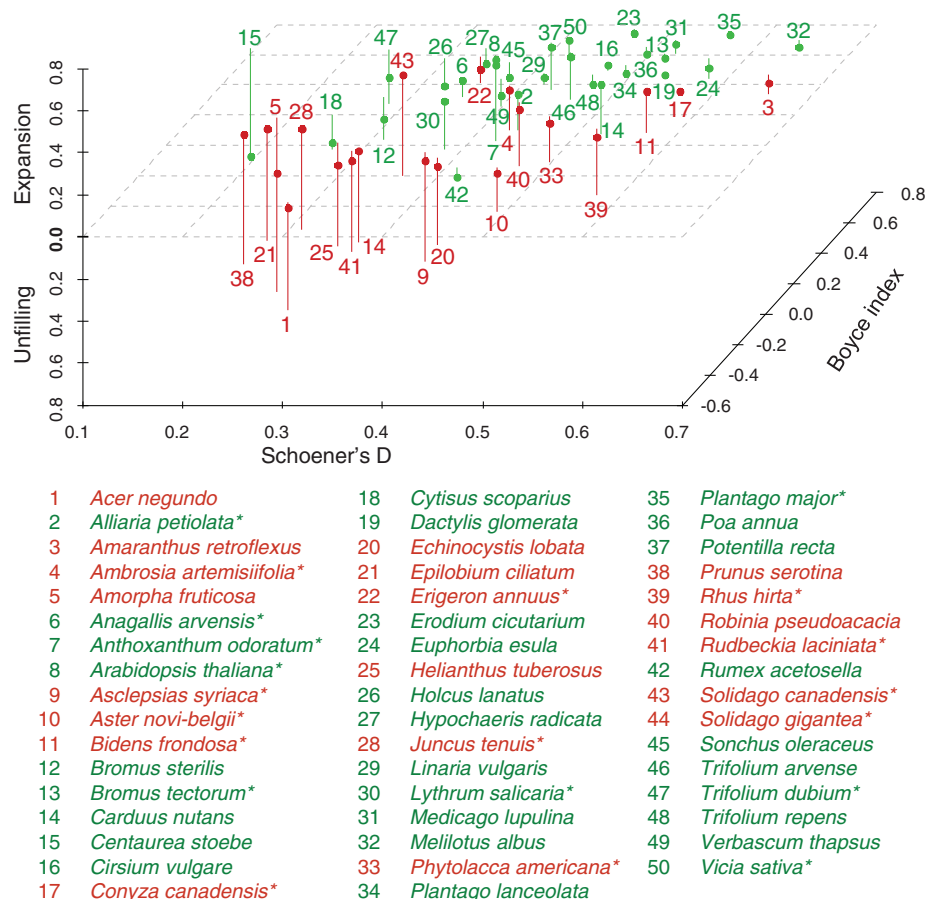
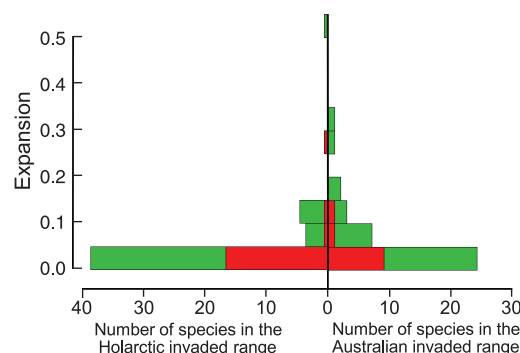


Fig. 1. Niche changes between native and invaded ranges in EU and NA. Vertical segments represent the magnitude of niche changes for each species. Extensions above and below the zero plane indicate expansion and unfilling, respectively. Intersections with the zero plane are shown with dots. Green (EU) and red (NA) colors indicate species origin. Niche-change indices are plotted over two niche-overlap indices, Schoener's D and the Boyce index evaluation of ENMs calibrated in the native range and projected onto analog climates in the invaded range. Asterisks denote species with a significant niche overlap between native and invaded range based on a similarity test.

Fig. 2. Expansion in Holarctic and Australian invaded ranges. The expansion index is analogous to the proportion of the invasive distribution in climate that is new to the species but available in the native range. NA and EU species origins are shown in red and green, respectively.



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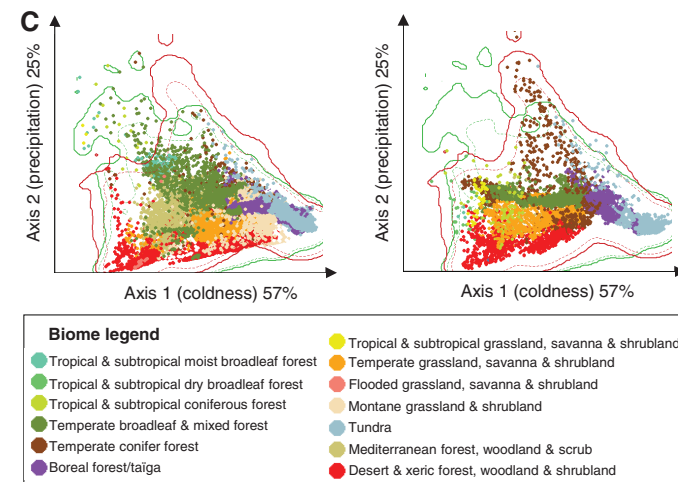
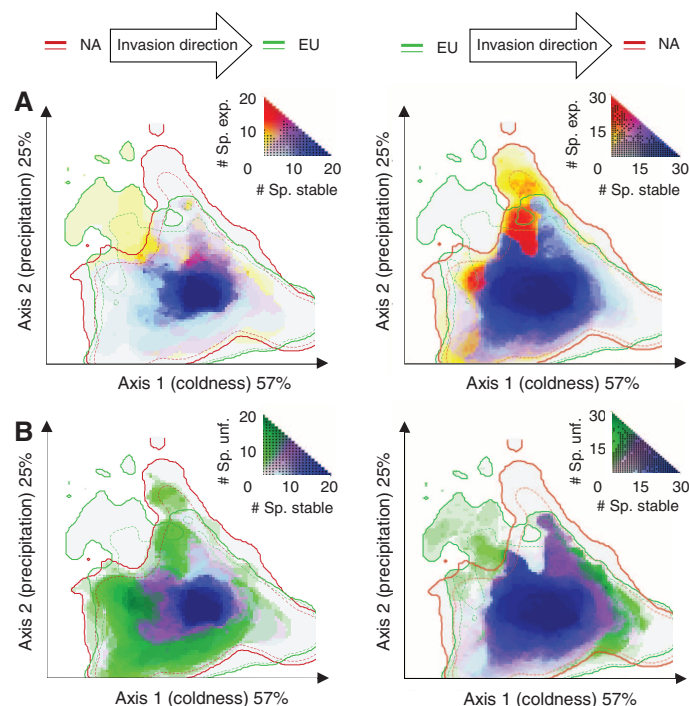


Fig. 3. Niche dynamic between native and invaded ranges in Holarctic environmental space depicted by the first two axes of a principal component analysis, calibrated on the entire range of conditions available in NA (red contour lines) and EU (green contour lines). Niche expansion, overlap, and unfilling situations are stacked in the environmental space for each species. Bidimensional color keys represent the number of species showing expansion [gray to red, (A)], unfilling [gray to green, (B)], and overlap [gray to blue, (A) and (B)]. Occupied color classes are shown by black dots. (C) Distribution of biomes in the invaded environmental space.

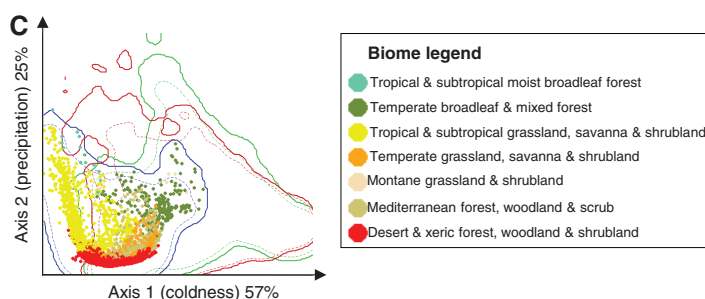
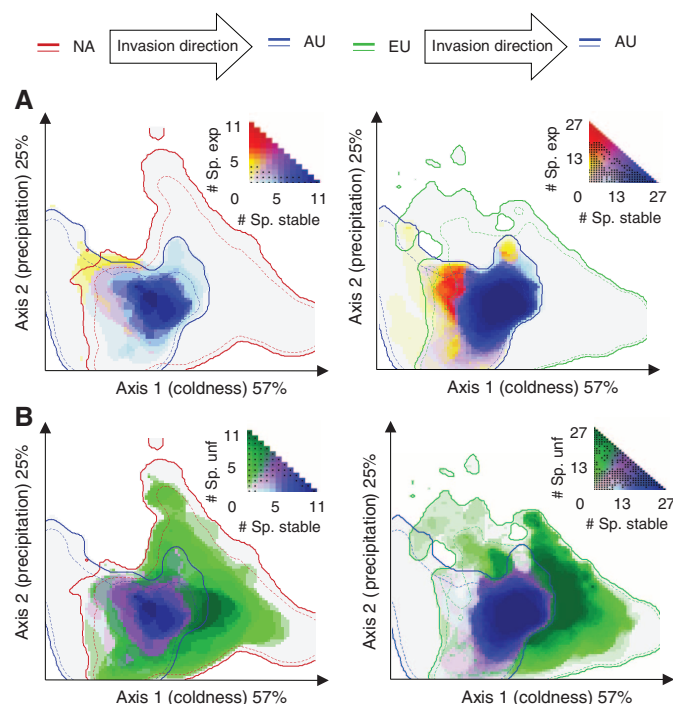


Fig. 4. (A to C) Niche dynamic between native and invaded ranges in Australian environmental space. Same as in Fig. 3, except realized environment in AU is additionally represented (blue contour lines), and (C) represents biomes distribution in AU.

terrestrial alien angiosperms (tables S1 and S2). Plants are appropriate for this test because their distributions are largely limited by climatic factors (22), a necessary condition to assess niche conservatism. Our sample includes many of the major plant invaders between North America (NA) and Eurasia (EU) and also many of the most anciently introduced EU species in NA. The reciprocal comparison of EU and NA invaders provides an important test of niche conservatism,

because EU and NA are the only pair of two large, separated land masses with a largely overlapping climate space and a long history of reciprocal anthropogenic exchanges of floras (23, 24). When available, the distribution of these species in Australia (AU) (table S3), where none is native, was used to provide additional, independent insights into patterns of niche filling when climatic availability, although partly overlapping, is overall very different from the native range. Geograph-

ical distributions (resolution = 0.5°, ~50 km) were projected onto climate space following a niche quantification framework correcting for species densities and climatic availability in both the native and invaded range (12, 21). This approach tests for niche conservatism and robustly quantifies the amount of niche overlap, expansion, and unfilling in the invaded range.

We find little evidence of niche expansion associated with the invasion of new regions. Our

results for the Holarctic reveal that, although levels of niche overlap among species vary between 17 and 64% (Fig. 1 and table S5), niche conservatism is observed for 46% (23) of species between the native and invaded range in EU and NA (similarity test with a significance level ≤ 0.05) (Fig. 1 and table S5). NA species show a higher propensity toward niche similarity (13 out of 20 species). In contrast to comparisons between EU and NA, niche similarity tests for AU are significant for all species (table S6), despite more pronounced climatic differences between AU and both EU and NA, respectively, than between EU and NA. This indicates that in AU, Holarctic invasive species remain in Holarctic climates and are rarely found in new climates. In other words, when considering the available climate in the invaded range, species colonize climatic conditions close to the ones colonized in their native range.

Further differentiating nonoverlap situations into cases of unfilling or expansion reveals that in the Holarctic, only 14% (7) of the studied species show more than 10% expansion, with only one outlier species—spotted knapweed (*Centaurea stoebe*)—showing >50% expansion (Figs. 1 and 2 and table S5). Previous studies also reported an important niche shift for this species (4), possibly caused by evolutionary (25) and/or ecological processes (15), notably through hybridization (4, 26) and enhanced competitive strength in the invaded range (27). There is also evidence of genetic admixing (repeated introductions or hybridization) and reduced impacts of competitors and enemies in many of the other studied species [e.g., (26, 28–30)], but these species did not show any major niche expansion, indicating that these mechanisms do not necessarily lead to niche expansion. Niche unfilling is a more widespread phenomenon with 48% (24) of species showing more than 10% of their native niche unfilled in the invasive range (Figs. 1 and 3). Patterns in AU confirm these Holarctic findings; i.e., niche expansion is uncommon compared to unfilling (Figs. 2 and 4, fig. S4, and table S6).

The biogeographical origin of the species provides further insights into niche comparisons between native and invaded ranges (Figs. 3 and 4). In general, EU species show less niche unfilling and more expansion in NA and AU than do NA species in EU and AU, thus mirroring biogeographical patterns of invasibility, which show higher invasion rates in NA compared with EU (31). Differences in the geographic arrangement of EU versus NA could account for the difference in niche unfilling. In particular, climate varies on a shorter distance along latitudinal gradients in NA than in EU and may allow more rapid expansion into different climates in NA (32). However, this does not explain why EU species also show less niche unfilling in AU than NA species. Biome conservatism, frequent across long evolutionary time scales (33) and highly expected in the case of invasive species (13), may further explain niche differences between areas differing in biome availability (Figs. 3 and 4). In NA and AU, EU species

expansions occur toward hotter and drier niche limits, corresponding in NA to the median climatic conditions of temperate coniferous forests, which are mostly absent in EU (Fig. 3). The lower prevalence of niche unfilling in EU species may relate to the longer history of weed selection in human-disturbed landscapes in Europe and earlier colonization paths from Europe to other continents (23, 24). However, when testing the effect of minimum residence time on niche expansion, overlap, unfilling, and total change magnitude, we found no significant effect (table S5), suggesting that other drivers, such as human-mediated propagule pressure, likely prevail. Movement of human settlements was far more important from EU toward NA and AU than the opposite (31), as shown by higher numbers of Eurasian invaders worldwide (24); this could explain less unfilling among EU species.

Our findings have implications for anticipating biological invasions, as they suggest that ENMs remain reasonable tools to predict invasions from the native range if study areas have comparable environments, at least with regard to the biologically relevant variables. This was indeed the rule in most of our species and thus is likely to also apply to many other terrestrial alien invasive plants. To illustrate this, we built ENMs for each species' native distribution. On average, the models reveal a fair transferability, with only a minority of poor predictions in the invaded range (eight NA species and two EU species) based on the Boyce index (B) (12). As expected, we found a positive correlation between B and the niche overlap D, as well as negative correlations between B and total niche changes (fig. S6). Interestingly, similar results are obtained when comparing niche metrics with ENM predictions calibrated on the analog climates between EU and NA or on the whole climate (fig. S7). The use of this approach to niche comparison (21) as a complement to ENMs thus remains important because it allows disentangling of disequilibrium situations, such as niche expansion or partial filling, in analog climates (Fig. 1).

Our findings that climatic niche shifts are rare among terrestrial plant invaders between their native and introduced ranges parallel results from a recent study showing that increases in species' abundance are rare between ranges (34). We found only a few plant invaders (such as spotted knapweed) that show an important proportion of their invaded range outside their native niche, possibly resulting from ecological and/or evolutionary changes, although we cannot exclude dispersal limitation in the native range as a possible contributing factor. Conversely, most reported niche differences are probably caused by partial filling of the native niche in the invaded range. Recognizing that some cases of true niche change do exist, further assessments should seek to understand strategies that have allowed these particular alien invasive species to expand their niches dramatically, with possible implications for biocontrol (35). Although our study focused on Holarctic plant

invaders, we included a wide range of plants, ranging from trees to herbs. It would be particularly interesting to use the same framework to test whether the same pattern is found in other organisms, especially in aquatic plants, as some of these are known to have a very large invaded range compared with their native one (36). Finally, our study specifically tested for niche change between geographic regions, but our general finding of niche conservatism also supports an important role for ENMs in assessments of species vulnerability to climate change over time (1).

References and Notes

- P. B. Pearman, A. Guisan, O. Broennimann, C. F. Randin, *Trends Ecol. Evol.* **23**, 149 (2008).
- J. J. Wiens, C. H. Graham, *Annu. Rev. Ecol. Syst.* **36**, 519 (2005).
- M. Vilà *et al.*, *Front. Ecol. Environ.* **8**, 135 (2010).
- O. Broennimann *et al.*, *Ecol. Lett.* **10**, 701 (2007).
- R. V. Gallagher, L. J. Beaumont, L. Hughes, M. R. Leishman, *J. Ecol.* **98**, 790 (2010).
- M. C. Fitzpatrick, J. F. Weltzin, N. J. Sanders, R. R. Dunn, *Glob. Ecol. Biogeogr.* **16**, 24 (2007).
- K. A. Medley, *Glob. Ecol. Biogeogr.* **19**, 122 (2010).
- C. Lauzeral *et al.*, *Glob. Ecol. Biogeogr.* **20**, 407 (2011).
- J. M. Alexander, P. J. Edwards, *Oikos* **119**, 1377 (2010).
- B. Leung *et al.*, *Proc. Biol. Sci.* **269**, 2407 (2002).
- A. Guisan, W. Thuiller, *Ecol. Lett.* **8**, 993 (2005).
- Materials and methods are available as supporting material on Science Online.
- W. Thuiller *et al.*, *Glob. Change Biol.* **11**, 2234 (2005).
- J. N. Klironomos, *Nature* **417**, 67 (2002).
- J. L. Hierro, D. Villarreal, O. Eren, J. M. Graham, R. M. Callaway, *Am. Nat.* **168**, 144 (2006).
- S. Lavergne, J. Molofsky, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 3883 (2007).
- C.-Y. Xu *et al.*, *Ecol. Lett.* **13**, 32 (2010).
- E. Welk, *Ecol. Model.* **179**, 551 (2004).
- D. L. Warren, R. E. Glor, M. Turelli, *Evolution* **62**, 2868 (2008).
- L. Mandle *et al.*, *PLoS ONE* **5**, e15297 (2010).
- O. Broennimann *et al.*, *Glob. Ecol. Biogeogr.* **10.1111/j.1466-8238.2011.00698.x** (2011).
- F. I. Woodward, *Climate and Plant Distribution* (Cambridge Univ. Press, Cambridge, 1987).
- A. W. Crosby, *Ecological Imperialism: The Biological Expansion of Europe, 900-1900 (Studies in Environment and History)* (Cambridge Univ. Press, Cambridge, 1986).
- T. Seipel *et al.*, *Glob. Ecol. Biogeogr.* **21**, 236 (2012).
- U. A. Treier *et al.*, *Ecology* **90**, 1366 (2009).
- A. C. Blair, R. A. Hufbauer, *Evol. Appl.* **3**, 40 (2010).
- R. M. Callaway *et al.*, *Ecology* **92**, 2208 (2011).
- W. Durka, O. Bossdorf, D. Prati, H. Auge, *Mol. Ecol.* **14**, 1697 (2005).
- S. J. Novak, R. N. Mack, *Bioscience* **51**, 114 (2001).
- M. Pairon *et al.*, *Ann. Bot. (London)* **105**, 881 (2010).
- T. R. Seastedt, P. Pyšek, *Annu. Rev. Ecol. Syst.* **42**, 133 (2011).
- M. Rejmánek, *Austral. Ecol.* **25**, 497 (2000).
- M. D. Crisp *et al.*, *Nature* **458**, 754 (2009).
- J. Finn *et al.*, *Ecol. Lett.* **14**, 274 (2011).
- H. Müller-Schärer, U. Schaffner, T. Steinger, *Trends Ecol. Evol.* **19**, 417 (2004).
- I. W. Forno, *Aquat. Bot.* **17**, 71 (1983).

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Supporting Online Material
www.sciencemag.org/cgi/content/full/335/6074/1344/DC1
Materials and Methods
SOM Text
Figs. S1 to S10

Tables S1 to S9
References (37–65)

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The Path from β -Carotene to Carlactone, a Strigolactone-Like Plant Hormone

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Strigolactones, phytohormones with diverse signaling activities, have a common structure consisting of two lactones connected by an enol-ether bridge. Strigolactones derive from carotenoids via a pathway involving the carotenoid cleavage dioxygenases 7 and 8 (CCD7 and CCD8) and the iron-binding protein D27. We show that D27 is a β -carotene isomerase that converts all-*trans*- β -carotene into 9-*cis*- β -carotene, which is cleaved by CCD7 into a 9-*cis*-configured aldehyde. CCD8 incorporates three oxygens into 9-*cis*- β -apo-10'-carotenal and performs molecular rearrangement, linking carotenoids with strigolactones and producing carlactone, a compound with strigolactone-like biological activities. Knowledge of the structure of carlactone will be crucial for understanding the biology of strigolactones and may have applications in combating parasitic weeds.

The biosynthetic carotenoid pathway provides precursors for smaller, physiologically important compounds such as the plant hormone abscisic acid (1), the vertebrate morphogen retinoic acid (2), and the fungal pheromone trisporic acid (3), which originate from oxidative cleavage of C-C double bonds in carotenoid backbones (4–7). The derivative compounds, named apocarotenoids (see fig. S1 for nomenclature and structures), may undergo structural modifications concealing their origin. Strigolactones, a prominent example of such compounds, are a group of metabolites with a common C₁₉ structure (e.g., 5-deoxystrigol, Fig. 1A) consisting of a tricyclic lactone (A, B, and C rings) connected via an enol ether bridge to a second lactone (D ring) (8). Several lines of evidence demonstrate that strigolactones derive from carotenoids. A putative biosynthetic pathway has been proposed, leading from a C₁₅ apocarotenal to the ABC C₁₄ skeleton, which is then coupled to a butenolide group (D ring) of unknown origin (9).

Released by plant roots, strigolactones trigger seed germination of root parasitic weeds such as witchweeds (*Striga* spp.) (10) and can induce hyphal branching in arbuscular mycorrhizal fungi, an essential step in establishing the symbiosis

(11). In plants, strigolactones inhibit tillering and shoot branching. High tillering (more branching) is observed in plant mutants disrupted in *Carotenoid Cleavage Dioxygenase 7* (CCD7) and CCD8, in *Dwarf27* (D27) encoding an iron-binding polypeptide with unknown catalytic properties, or in a cytochrome P450 gene represented by *More Axillary Growth 1* (MAX1) from

Arabidopsis thaliana (8, 12–17). The application of the synthetic strigolactone GR24 restored the wild-type tillering/branching phenotype of these mutants, suggesting the involvement of CCD7, CCD8, D27, and MAX1 in strigolactone biosynthesis (8, 12–17).

CCDs are nonheme iron enzymes that cut C-C double bonds by incorporating a dioxygen, yielding carbonyl products (4–7). It was supposed that CCD7 from *Arabidopsis* (AtCCD7) cleaves all-*trans*- β -carotene (C₄₀; Fig. 1A) into all-*trans*- β -apo-10'-carotenal (C₂₇) that is then further shortened by AtCCD8 to the C₁₈ ketone β -apo-13-carotenone (Fig. 1B) (18), a biologically active compound affecting the growth of root hairs in plants (19). The sequential activity of the two cleavage enzymes was deduced from the coexpression of AtCCD7 and AtCCD8 in *Escherichia coli* strains accumulating all-*trans*- β -carotene (18) and confirmed by in vitro studies showing the formation of β -apo-13-carotenone from all-*trans*- β -apo-10'-carotenal by CCD8 enzymes from different plant species (20). However, the structure of β -apo-13-carotenone has little in common with that of strigolactones, and treatment of rice *ccd8* mutant with this compound did not restore the wild-type tillering phenotype (fig. S2).

We used an in vitro approach to investigate the activities of CCD7 and CCD8 from *Arabidopsis*,

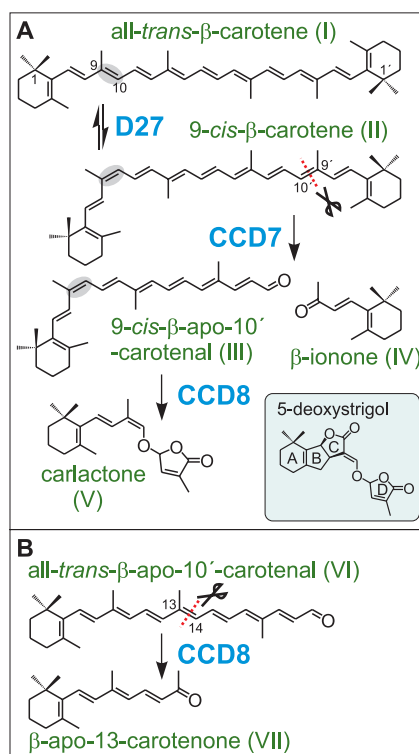


Fig. 1. The pathway to carlactone. (A) D27 catalyzes the isomerization of the C9-C10 double bond (shaded) in all-*trans*- β -carotene (I; C₄₀), leading to 9-*cis*- β -carotene (II) that is cleaved by CCD7 at the C9', C10' position into 9-*cis*- β -apo-10'-carotenal (C₂₇; III) and β -ionone (C₁₃; IV). A single enzyme, CCD8, converts III to carlactone (V) that already contains the D ring and the enol ether bridge of strigolactones such as 5-deoxystrigol (inset). (B) CCD8 also catalyzes a known CCD reaction by converting all-*trans*- β -apo-10'-carotenal into β -apo-13-carotenone. However, this reaction is slower than that with the *cis* isomer by about a factor of 10.

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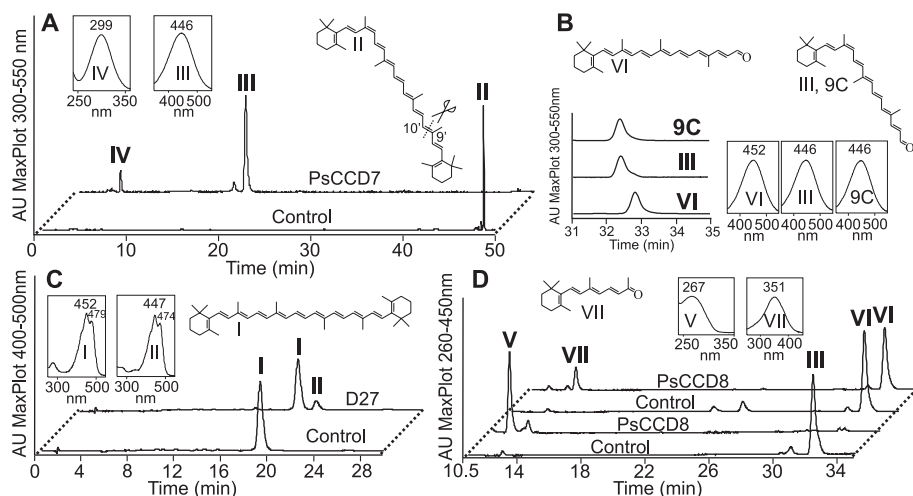
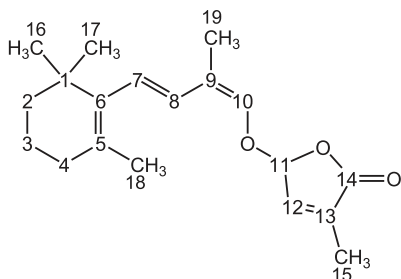


Fig. 2. HPLC analyses of in vitro incubations with PsCCD7, D27, and PsCCD8. (A) Incubation of PsCCD7 with 9-*cis*- β -carotene (II) yielded 9-*cis*- β -apo-10'-carotenal (III) and β -ionone (IV). (B) Confirming its 9-*cis* configuration, compound III coeluted with the reference compound 9-*cis*- β -apo-10'-carotenal (9C) 0.5 min earlier than with all-*trans*- β -apo-10'-carotenal (VI). (C) Incubation of purified D27 with all-*trans*- β -carotene (I) led to the formation of 9-*cis*- β -carotene (II). (D) PsCCD8 converted 9-*cis*- β -apo-10'-carotenal (III) into an apocarotenoid (V) different from β -apo-13-carotenone (VII). The samples were analyzed using separation systems A (A), B [(B) and (D)], and C (C) (see supporting online material). UV-Vis spectra are depicted in the insets. AU, arbitrary units.

Table 1. ^1H and ^{13}C NMR data of compound V: experimental ^1H and ^{13}C chemical shifts, ^1H - ^{13}C heteronuclear multiple bond correlations (HMBC), and relevant ^1H - ^1H nuclear Overhauser enhancements (NOE) data for compound V dissolved in and referenced to CD_2Cl_2 (^1H , 5.32 ppm; ^{13}C , 54.0 ppm).

	^1H	^{13}C	^1H - ^{13}C HMBC	1D ^1H - ^1H -NOE
1	-	34.37		
2	1.46 (2 H, m)	39.86	C-1, C-3, C-4, C-6, C-16 and C-17	
3	1.61 (2 H, m)	19.59	C-1, C-2, C-4 and C-5	
4	2.00 (2 H, br t, 6.5 Hz)	33.24	C-2, C-3, C-5, C-6 and C-18	
5	-	129.38		
6	-	138.18		
7	6.05 (1 H, d, 16.2 Hz)	127.47	C-5 and C-9	H-16, H-17 and H-19
8	6.46 (1 H, d, 16.4 Hz)	127.47	C-6, C-9, C-10 and C-19	no NOE for H-10
9	-	117.62		
10	6.20 (1 H, s)	138.21	C-8, C-9, C-11 and C-19	H-11 and H-19 no NOE for H-8
11	5.97 (1 H, br p, 1.5 Hz)	100.65	C-10	H-10 and H-12
12	6.90 (1 H, br p, 1.5 Hz)	142.67		H-11 and H-15
13	-	135.03		
14	-	171.52		
15	1.95 (3 H, t, 1.5 Hz)	10.71	C-12, C-13 and C-14	H-12
16, 17	1.00 (6H, s)	29.02	C-1, C-2, C-6, C-7, C-16 and C-17	
18	1.69 (3H, s)	21.69	C-4, C-5, C-6, C-7, C-16 and C-17	
19	1.73 (3H, d, 1.2 Hz)	14.39	C-6, C-7, C-8 and C-9	



Pisum sativum, and *Oryza sativa*. Carotenoids occur in different *cis* configurations, a feature that may determine reaction specificities (1, 21). Indeed, we show that the *cis* configuration of the C9-C10 double bond in β -carotene is decisive for the course of the reactions in strigolactone biosynthesis (Fig. 1) and that this pathway is characterized by the function of a single enzyme, CCD8, in converting a 9-*cis*-configured apocarotenal into a strigolactone-like compound (Fig. 1A).

As shown by high-performance liquid chromatography (HPLC) analysis, soluble fractions of *E. coli* cells expressing *P. sativum* CCD7 (PsCCD7) fused with thioredoxin (Fig. 2A), as well as purified PsCCD7 fused with glutathione *S*-transferase (GST; fig. S3), cleaved 9-*cis*- β -carotene (II, C_{40}) into 9-*cis*- β -apo-10'-carotenal (III, C_{27}) and β -ionone (IV, C_{13}). All other β -carotene isomers tested, including all-*trans*-, 13-*cis*-, and 15-*cis*- β -carotene, were not converted (fig. S4, B to D). The products were identified by gas chromatography and liquid chromatography coupled to mass spectrometry (GC-MS and LC-MS) (fig. S5) and according to their ultraviolet/visible (UV/Vis) absorption spectra. The configuration of β -apo-10'-carotenal (III) was confirmed by HPLC comparison (Fig. 2B) with the reference compounds all-*trans*- β -apo-10'-carotenal (VI) and 9-*cis*- β -apo-10'-carotenal (9C). *Arabidopsis* and *O. sativa* thioredoxin-CCD7 crude preparations exhibited the same activity (fig. S4A). Thus, CCD7 enzymes are stereospecific for the 9-*cis* configuration and cleave the C9'-C10' double bond in the *trans* moiety of 9-*cis*- β -carotene (Fig. 1A).

In plants, the carotenoid biosynthetic pathway provides β -carotene in the all-*trans* configuration (22), because the enzyme forming β -carotene, lycopene- β -cyclase, acts as a selectivity filter for the all-*trans* isomer (23). *Cis* isomers may arise nonspecifically from photo-60 isomerization (24) or thermo-isomerization (25). Alternatively, a specific all-*trans*/9-*cis*- β -carotene isomerase may be postulated to divert the carotenoid pathway into strigolactone biosynthesis by forming the authentic CCD7 substrate. We tested whether D27, a strigolactone biosynthetic enzyme with hitherto unknown catalytic properties (26), exerts this function. In vitro incubation of all-*trans*- and 9-*cis*- β -carotene with purified protein demonstrates that D27 is indeed a carotene isomerase that reversibly converts all-*trans*- β -carotene into the CCD7 substrate 9-*cis*- β -carotene (Fig. 2C; see also fig. S6). These data suggest that D27 is the first all-*trans*/9-*cis*- β -carotene isomerase reported so far and indicates that D27 is the enzyme providing the substrate for CCD7 in the course of strigolactone biosynthesis (Fig. 1A).

The configuration of the C9-C10 double bond of β -apo-10'-carotenal determines the nature of the product formed by CCD8. Incubation of thioredoxin-PsCCD8 crude preparations with the CCD7 product 9-*cis*- β -apo-10'-carotenal yielded an unknown compound (V, Fig. 2D) with an absorption maximum at 267 nm, lower than that of β -apo-13-carotenone (VII, Fig. 2D) reported

to be formed by CCD8 from all-*trans*- β -apo-10'-carotenal (19, 20). Incubations of thioredoxin-OsCCD8b and thioredoxin-AtCCD8 led to the same results, demonstrating the generality of the finding that CCD8 enzymes use both β -apo-10'-carotenal isomers as substrates but can produce different products (Fig. 1 and fig. S7, A and B). Time courses of product formation (fig. S7C) show that the conversion of the *cis* substrate is faster than that of the *trans* substrate by a factor of ~ 10 . We conclude that the conversion of the *cis* substrate into compound V is not a side reaction.

The structure of compound V was elucidated using electrospray ionization mass spectrometry (ESI-MS) and 1D and 2D nuclear magnetic resonance (NMR) spectroscopy, exploiting NMR reference data (27) and ^1H - ^{13}C -chemical shift prediction (27). The NMR data are summarized in Table 1. Both the ESI-MS data ($[\text{M}+\text{H}]^+$: 303.19, $\text{C}_{19}\text{H}_{26}\text{O}_3$; fig. S8) and chemical shifts at C9, C10, and C11 pointed to oxygen as the linker between C10 and C11. This finding was

corroborated by taking advantage of 2D ^1H - ^{13}C heteronuclear multiple bond correlation and 1D ^1H - ^1H nuclear Overhauser enhancement NMR spectroscopy (figs. S9 to S15). ^1H and ^{13}C chemical shift prediction (28) confirmed the proposed structure. Thus, compound V [IUPAC nomenclature: 2',3'Z,4'5'E-3-methyl 5-(3'-methyl-1'-oxa-penta-2',4'-dienyl-5'-(2'',6'',6''-trimethylcyclohex-1'-en-1'-yl))-2(5H)-furanone] is structurally similar to strigolactones (Fig. 1A), including the number of C atoms and the presence of a lactone. We have named compound V carlactone (Fig. 1A), reflecting its origin from β -carotene and its relationship to strigolactones.

Carlactone was also produced when 9-*cis*- β -apo-10'-carotenal was incubated with purified PsCCD8, instead of lysates of overexpressing *E. coli* cells, and in the absence of any added cofactor (fig. S16). Thus, the C skeleton of carlactone derives entirely from 9-*cis*- β -apo-10'-carotenal, and CCD8 alone is capable of catalyzing all the reactions needed to form carlactone from this substrate, including the introduction of three oxygens and intramolecular rearrangement. A hypothetical mechanism for the conversion of 9-*cis*- β -apo-10'-carotenal into carlactone is presented in fig. S17.

We first performed each assay with each enzyme individually. Strigolactone biosynthesis was initiated by the D27-mediated isomerization of all-*trans*- to 9-*cis*- β -carotene, followed by the CCD7-catalyzed formation of 9-*cis*- β -apo-10'-carotenal that was converted by CCD8 into carlactone (Fig. 1A). To investigate the effect of interactions between enzymes, we then combined PsCCD7 and PsCCD8 in vitro. No products were detected with all-*trans*- β -carotene as a substrate, but 9-*cis*- β -carotene was converted into carlactone (fig. S18A). Addition of the isomerase D27 to PsCCD7 and PsCCD8 allowed the formation of carlactone from all-*trans*- β -carotene (fig. S18B).

The absence of carlactone in *d27*, *ccd7*, and *ccd8* mutants is the reason for the more-branching, high-tillering phenotype observed. We applied carlactone produced in vitro to the rice *d27*, *ccd7* (*htd-1*), and *ccd8* (*d10*) mutants and, as a control, to the wild type and to *d3*, a high-tillering strigolactone signal perception mutant (17). After treatment for 28 days, tiller numbers of *d27*, *htd-1*, and *d10* mutants were reduced (*d27* from 5.3 to 3; *htd-1* from 6 to 2.3; *d10* from 5.7 to 1.3), whereas wild-type and *d3* tiller numbers were not affected (wild type, 2; *d3*, 4.5) (Fig. 3, A and B). The activity of carlactone in restoring the wild-type tillering phenotype in *d27*, *ccd7*, and *ccd8* mutants indicates that this compound is a likely precursor of strigolactones. Only a few reactions—dioxygenation followed by a dehydrogenation and simultaneous closure of the B and C rings (fig. S19B)—would be required to convert carlactone into 5-deoxystriol. This appears feasible in view of a recent paper that shows the chemical synthesis of the strigolactone B/C ring system from a linear precursor in a single

acid-catalyzed step (29). However, we have not been able to detect carlactone in plants.

Carlactone can induce seed germination of parasitic plants. We treated *Striga hermonthica* seeds with various concentrations of carlactone and with GR24 as a positive control. Carlactone, although not as effective as GR24, did induce seed germination in a concentration-dependent manner (Fig. 3C). The germination-promoting activity of carlactone shows that the enone moiety of the C ring of strigolactones (Fig. 1A) is not mandatory, contradicting previous suggestions (30, 31).

The comparatively simple structure of carlactone opens up new possibilities for designing strigolactone/carlactone analogs that could be applied to induce suicidal germination, a promising tool to combat root parasitic weeds that represent severe agricultural pests worldwide (32). Knowledge of the carlactone structure will greatly assist in identifying the bioactive compound(s) that regulate shoot branching and in elucidating further steps in strigolactone biosynthesis, including the activities of MAX1 (8, 14–17).

References and Notes

- S. H. Schwartz, B. C. Tan, D. A. Gage, J. A. Zeevaert, D. R. McCarty, *Science* **276**, 1872 (1997).
- J. von Lintig, *Annu. Rev. Nutr.* **30**, 35 (2010).
- H. R. Medina, E. Cerdá-Olmedo, S. Al-Babili, *Mol. Microbiol.* **82**, 199 (2011).
- F. Bouvier, J. C. Isner, O. Dogbo, B. Camara, *Trends Plant Sci.* **10**, 187 (2005).
- A. R. Moise, J. von Lintig, K. Palczewski, *Trends Plant Sci.* **10**, 178 (2005).
- M. E. Auldridge, D. R. McCarty, H. J. Klee, *Curr. Opin. Plant Biol.* **9**, 315 (2006).
- M. H. Walter, D. Strack, *Nat. Prod. Rep.* **28**, 663 (2011).
- X. Xie, K. Yoneyama, K. Yoneyama, *Annu. Rev. Phytopathol.* **48**, 93 (2010).
- R. Matusova et al., *Plant Physiol.* **139**, 920 (2005).
- C. E. Cook, L. P. Whitchard, B. Turner, M. E. Wall, G. H. Egle, *Science* **154**, 1189 (1966).
- K. Akiyama, K. Matsuzaki, H. Hayashi, *Nature* **435**, 824 (2005).
- M. Umehara et al., *Nature* **455**, 195 (2008).
- V. Gomez-Roldan et al., *Nature* **455**, 189 (2008).
- O. Leyser, *Dev. Cell* **15**, 337 (2008).
- O. Leyser, *Plant Cell Environ.* **32**, 694 (2009).
- C. A. Beveridge, J. Kyozuka, *Curr. Opin. Plant Biol.* **13**, 34 (2010).
- Y. Wang, J. Li, *Curr. Opin. Plant Biol.* **14**, 94 (2011).
- S. H. Schwartz, X. Qin, M. C. Loewen, *J. Biol. Chem.* **279**, 46940 (2004).
- M. Schlicht et al., *Plant J.* **55**, 709 (2008).
- A. Alder, I. Holdermann, P. Beyer, S. Al-Babili, *Biochem. J.* **416**, 289 (2008).
- S. H. Schwartz, X. Qin, J. A. D. Zeevaert, *J. Biol. Chem.* **276**, 25208 (2001).
- D. DellaPenna, B. J. Pogson, *Annu. Rev. Plant Biol.* **57**, 711 (2006).
- Q. Yu, S. Ghisla, J. Hirschberg, V. Mann, P. Beyer, *J. Biol. Chem.* **286**, 8666 (2011).
- G. E. Bartley, P. A. Scolnik, P. Beyer, *Eur. J. Biochem.* **259**, 396 (1999).
- L. Zechmeister, *Cis-trans Isomeric Carotenoids, Vitamin A and Arylpolyenes* (Springer Verlag, Heidelberg, 1962), chap. V.
- H. Lin et al., *Plant Cell* **21**, 1512 (2009).
- G. Englert, in *Carotenoids, Vol. 18: Spectroscopy*, G. Britton, S. Liaen-Jensen, H. Pfander, Eds. (Birkhäuser, Basel, 1995), chap. 6.
- ACD Shift Prediction Software, Version 11.02, Advanced Chemistry Development Inc. (2008).

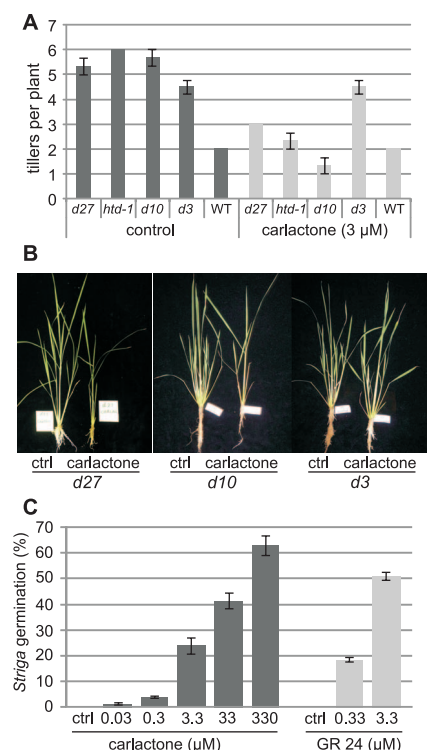


Fig. 3. Biological activity of carlactone. (A) Effect of carlactone on tillering in *d27*, *htd-1*, *d10*, *d3*, and the corresponding wild type (WT, Shikari). Application of carlactone (3 μM daily for 2 weeks) rescued the high-tillering phenotype of the strigolactone-deficient rice mutants, whereas *d3* and WT rice remained unaffected. (B) Photographs were taken after 30 days. (C) Germination rate of *S. hermonthica* seeds 2 days after treatment with different concentrations of carlactone, GR24, or water (ctrl). Data are means $\pm$ SD [(A), $n = 4$; (C), $n = 4$ with 50 to 100 seeds per replicate].

29. K. Chojnacka *et al.*, *Org. Biomol. Chem.* **9**, 5350 (2011).
30. K. Yoneyama, X. Xie, K. Yoneyama, Y. Takeuchi, *Pest Manag. Sci.* **65**, 467 (2009).
31. B. Zwanenburg, A. S. Mwakaboko, A. Reizelman, G. Anilkumar, D. Sethumadhavan, *Pest Manag. Sci.* **65**, 478 (2009).
32. C. Parker, *Pest Manag. Sci.* **65**, 453 (2009).

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European patent application (number 11 003 470.9) on carlactone and its applications.

Supporting Online Material

www.sciencemag.org/cgi/content/full/335/6074/1348/DC1

Materials and Methods

Figs. S1 to S19

References (33–38)

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Sexual Deprivation Increases Ethanol Intake in *Drosophila*

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The brain's reward systems reinforce behaviors required for species survival, including sex, food consumption, and social interaction. Drugs of abuse co-opt these neural pathways, which can lead to addiction. Here, we used *Drosophila melanogaster* to investigate the relationship between natural and drug rewards. In males, mating increased, whereas sexual deprivation reduced, neuropeptide F (NPF) levels. Activation or inhibition of the NPF system in turn reduced or enhanced ethanol preference. These results thus link sexual experience, NPF system activity, and ethanol consumption. Artificial activation of NPF neurons was in itself rewarding and precluded the ability of ethanol to act as a reward. We propose that activity of the NPF–NPF receptor axis represents the state of the fly reward system and modifies behavior accordingly.

Natural rewards and abused drugs affect the function of the brain's reward systems, and abnormal function of these brain regions is associated with addictive behavior (1–3). Some aspects of drug reward can be modeled in the genetically tractable fruit fly, *Drosophila melanogaster*. Flies exhibit complex addiction-like behaviors, including a lasting attraction for a cue that predicts ethanol intoxication (4) and a preference for consuming ethanol-containing food, even if made unpalatable (5). Here, we extend studies in the *Drosophila* model to incorporate the effect of social experiences, which can have long-lasting effects on behavior (6, 7).

We used two distinct sexual experiences to generate two cohorts of male flies. One cohort, rejected-isolated, was subjected to courtship conditioning (8); they experienced 1-hour sessions of sexual rejection by mated females, three times a day, for 4 days (Fig. 1A). Such conditioning suppresses future male courtship behavior, even toward receptive virgin females (9) (fig. S1). Flies in the mated-grouped cohort experienced 6-hour sessions of mating with multiple receptive virgin females (ratio 1:5) for 4 days. Flies from each cohort were then tested in a two-choice preference assay (10), in which they voluntarily choose to consume food with or without 15% ethanol supplementation (11). Results for the two co-

horts differed markedly. The value of the ethanol preference index (which when positive signifies attraction) was consistently higher for the rejected-isolated cohort (Fig. 1B). The experiences did not alter food consumption when tested in the absence of ethanol (fig. S2).

The rejected-isolated and mated-grouped cohorts differ in several respects in addition to sexual deprivation (lack of copulation) per se, including individual versus group housing, exposure to the social experience of rejection, and exposure to aversive chemosensory cues found on mated females. Several experiments were designed to determine which of these was the predominant contributor to the enhanced ethanol preference seen in rejected-isolated males (11). First, we compared males that differed in sexual experience but not in housing conditions—that is, mated and virgin males that were both group-housed. The virgin males showed higher ethanol preference, although in general not quite as high as rejected-isolated males. This argues that isolation is not the major explanation for the enhanced ethanol preference.

We next investigated ethanol preference in males that were sexually deprived (blocked from copulating) but not exposed to the social experience of rejection. For this purpose, males were exposed individually to decapitated virgin females on the same schedule as the rejected-isolated cohort, using a protocol that results in courtship suppression (9). These males, which experience neither rejection nor copulation, showed enhanced ethanol preference when compared to the mated-grouped cohort (Fig. 1C); the preference index was similar to that displayed by the rejected-isolated cohort. These results point to sexual

deprivation per se, rather than rejection, as the major factor influencing ethanol preference.

Finally, we sought to establish whether there was a role for the repellent chemosensory cue *cis*-vaccenyl acetate (cVA), which is found on the cuticle of mated but not virgin females (12). We compared males trained with either mated females (rejected-isolated) or decapitated virgin females (11). Both groups endured sexual deprivation (lack of copulation), but only the former was exposed to cVA. There was no difference in ethanol preference between these two cohorts (fig. S3A). There was also no difference between males exposed individually to biologically relevant concentrations of cVA (13) and vehicle-exposed controls (fig. S3B) (11). Together, these experiments point to sexual deprivation per se, rather than other factors, as the major contributor to enhanced ethanol preference.

To further test the strength of this conclusion, we divided a cohort of rejected-isolated males into two subgroups, one of which was left undisturbed, and the other of which was allowed to mate with virgin females for 2.5 hours immediately before testing. Ethanol preference was markedly lower in the rejected, then mated subgroup (Fig. 1E) compared to the subgroup that had only experienced rejection. Thus, the effects of sexual deprivation can be reversed by copulation, which is consistent with sexual deprivation being the major contributor to ethanol preference.

We focused on *Drosophila* neuropeptide F (NPF) as a potential mediator of the effects of sexual experience. The mammalian NPF homolog, neuropeptide Y [NPY (14)], regulates ethanol consumption (15), the NPF–NPF receptor (NPFR) system regulates acute ethanol sensitivity in *Drosophila* (16), and the *Caenorhabditis elegans* NPY receptor homolog NPR-1 regulates ethanol behaviors (17). Intriguingly, stressful experiences regulate mammalian NPY levels. These include restraint stress and early maternal separation in rodents and post-traumatic stress disorder in humans (18–20). However, a direct connection between social experience, NPY, and ethanol-related behaviors has not been established.

To investigate whether NPF mediates ethanol preference in *Drosophila*, we first compared NPF transcript levels in heads of males subjected to different sexual experiences: rejected-isolated, virgin-grouped, and mated-grouped (11). Rejected-isolated males showed the lowest transcript levels, virgin-grouped males showed higher levels, and mated-grouped males showed the highest

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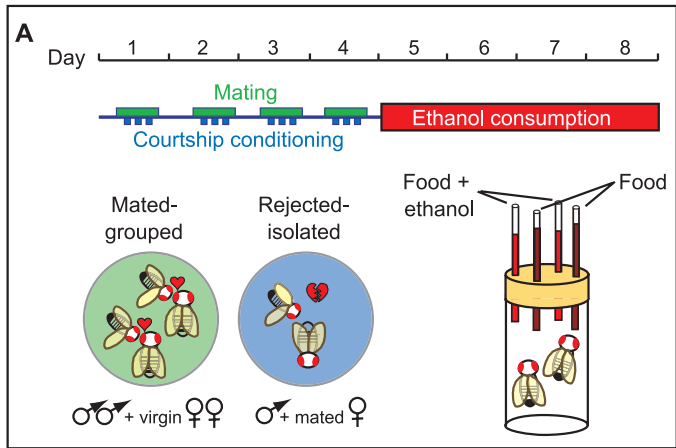
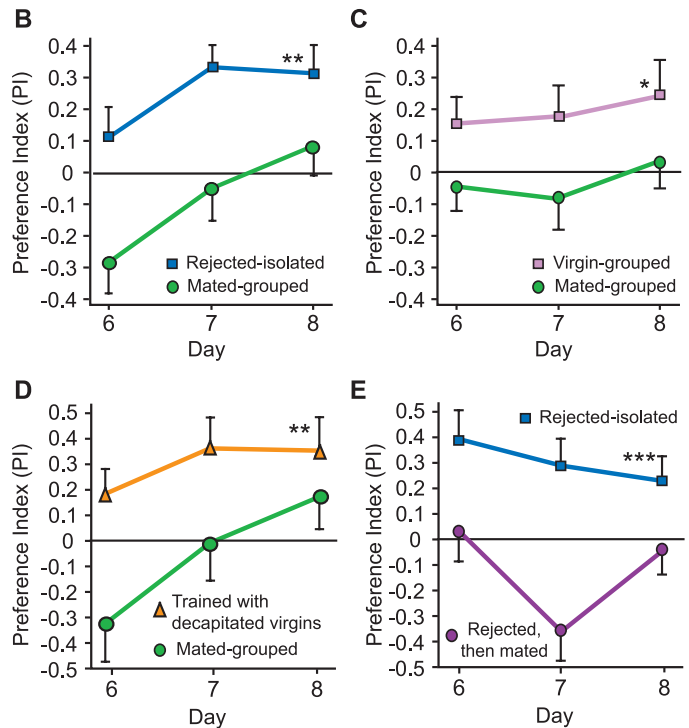


Fig. 1. Mating and chronic sexual deprivation have opposite effects on voluntary ethanol consumption. **(A)** Schematic of the behavioral assay. Virgin wild-type males were allowed to mate with virgin females (groups of 4 males and 20 females) for 6 hours daily (“mated-grouped”; green blocks) or were subjected to courtship conditioning for 1 hour, three times daily (“rejected-isolated”; blue squares). Training was repeated for 4 days, after which males were placed in vials where they could choose to feed from capillaries containing food solutions with (red) or without (brown) 15% ethanol (10). Ethanol consumption was measured on days 6 to 8. **(B)** Rejected-isolated males exhibited higher ethanol preference than mated-grouped males ($**P < 0.005$, $n = 12$). **(C)** Mated-grouped males showed lower ethanol preference than “virgin-grouped” males ($*P < 0.05$, $n = 12$). **(D)** Males conditioned with decapitated virgins showed enhanced ethanol preference compared to mated-grouped males ($**P < 0.01$, $n = 12$). **(E)** Mating reversed the effects of rejection on ethanol preference. Rejected-isolated males that were allowed to mate after the end of the last conditioning session showed lower ethanol preference than



similarly conditioned males that were left undisturbed ($**P < 0.001$, $n = 8$). Statistical analysis was carried out by two-way repeated-measures analysis of variance (ANOVA) with Bonferroni post tests; comparisons are between treatment groups across all days of the assay. Data shown are the mean $\pm$ SEM or mean $-$ SEM.

(Fig. 2A and fig. S4). Rejected-isolated males also showed markedly lower NPF protein levels than mated-grouped males by immunohistochemistry (Fig. 2, B to D).

To determine whether the inverse correlation between NPF levels and ethanol preference reflects a cause-and-effect relationship, we manipulated the NPF-NPFR system genetically. We first tested the effect of NPFR down-regulation by expressing an NPFR-specific short interfering RNA (*UAS-NPFR^{RNAi}*) pan-neuronally (using *elav-GAL4*). This manipulation significantly reduced ethanol preference in mated males, which have elevated NPF levels, but not in virgin males (Fig. 3, A and B). Second, we tested the effect of artificial activation of NPF neurons by expressing the heat-activated cation channel dTRPA1 (*21*) under *NPF-GAL4* control (22). There was no effect on ethanol preference when virgin males were tested at 20°C, when the channel is inactive, but there was aversion to ethanol-supplemented food at 29°C, when the channel is active (Fig. 3, C and D). An intermittent dTRPA1 activation protocol that more closely mimics our conditioning protocol produced similar aversion (fig. S5). These data suggest a causal relationship between sexual experience, NPF levels, and ethanol preference.

We propose that the activity of the NPF-NPFR system may be a neural representation of the state of the *Drosophila* reward system. If so, experiences that change NPF-NPFR activity

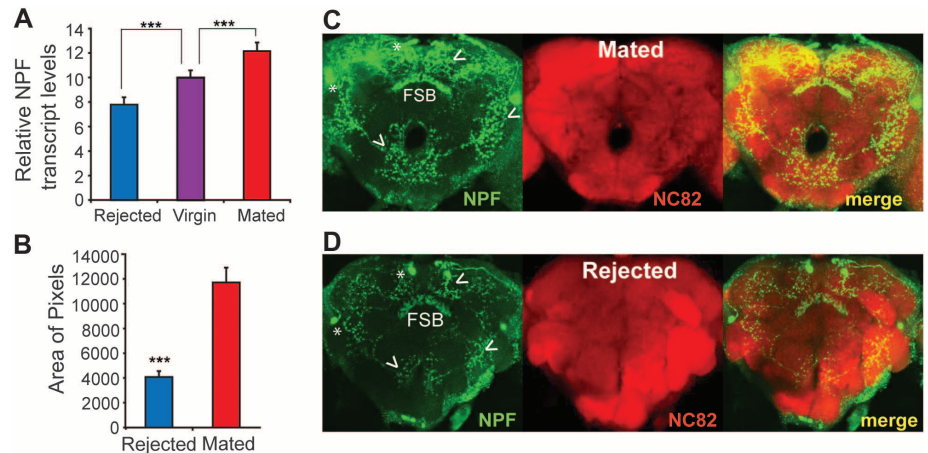


Fig. 2. Sexual experience regulates levels of NPF and NPF mRNA. **(A)** Total RNA extracted from heads of virgin, rejected, and mated males was analyzed for NPF mRNA levels by quantitative polymerase chain reaction (qPCR). NPF mRNA levels were reduced by sexual rejection and increased by mating ($***P < 0.001$ compared to virgin control, Dunnett’s test, $n = 3$ independent experiments). NPF transcript levels were normalized to *rp49* mRNA. **(B to D)** Effect of rejection on NPF protein abundance as determined by immunohistochemistry. **(B)** Quantitative analysis of overall NPF staining intensity in brains of rejected and mated males ($***P < 0.001$, t test). **(C and D)** Differential NPF staining in rejected and mated males was observed in all major regions of NPF expression (arrowheads). Asterisks denote the positions of NPF-expressing cell bodies (which are obscured by high levels of expression in mated males). FSB, fan-shaped body.

should promote behaviors that restore the system to its normal state. In this model, sexual deprivation would create an NPF deficit that increases reward-seeking behavior such as ethanol consump-

tion. Conversely, successful copulation would create a NPF surfeit that reduces reward seeking. This model predicts that mating and ethanol consumption should be rewarding (4, 5), that activa-

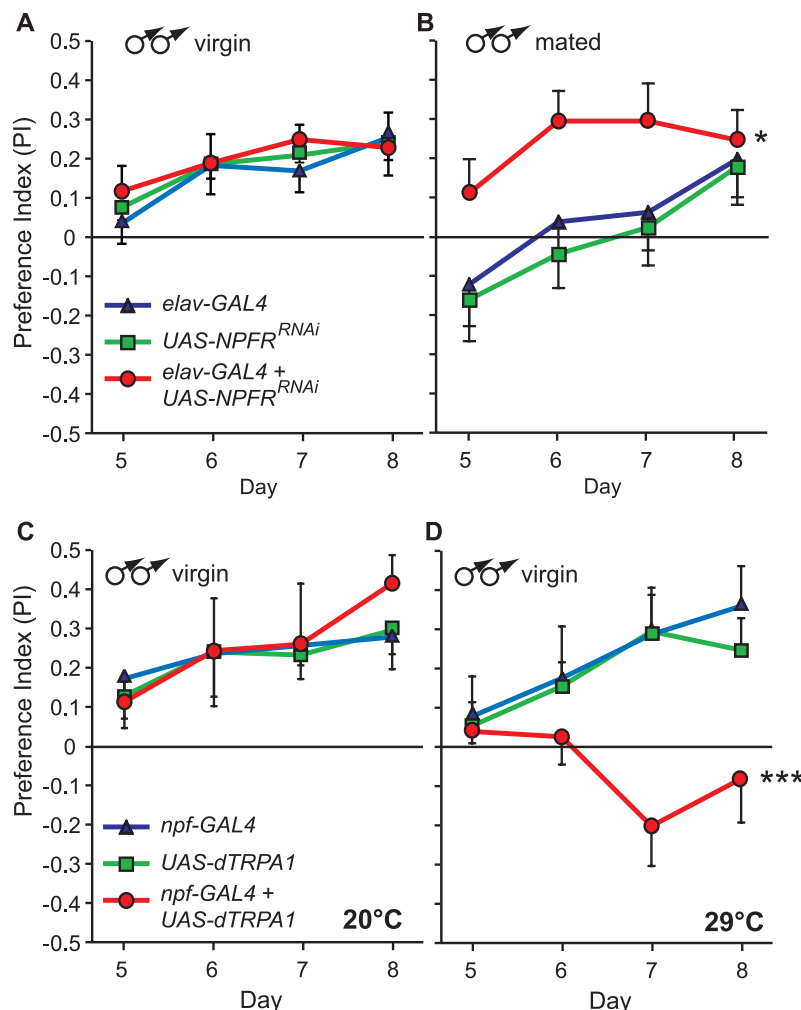


Fig. 3. NPF signaling regulates ethanol preference. (A and B) Expression of an NPFR RNA interference (*RNAi*) transgene (*UAS-NPFR^{RNAi}*) using a pan-neuronal driver (*elav-GAL4*) increased ethanol preference in mated males compared to the genetic controls carrying either transgene alone (B) (* $P < 0.05$, $n = 12$), but not in virgin males (A) ($P > 0.5$). (C and D) Activating NPF neurons reduced ethanol preference. Virgin males expressing dTRPA1 in NPF neurons (*NPf-GAL4 + UAS-dTRPA1*), and the genetic controls carrying either transgene alone, developed similar levels of ethanol preference at 20°C (C) when dTRPA1 is not active ($P > 0.05$, $n = 8$), but developed aversion to ethanol containing food at 29°C (D), when dTRPA1 is active (** $P < 0.001$, $n = 8$). Statistical analysis was carried out by two-way repeated-measures ANOVA with Bonferroni post tests; comparisons are between treatment groups across all days of the assay. Data are the mean $\pm$ or mean $-$ SEM (for clarity purposes).

tion of the NPF-NPFR pathway is rewarding per se, and that artificial activation of the NPF circuit will diminish ethanol reward-seeking behavior.

To test these predictions, we used a series of conditioning assays in which male flies were trained to associate the proposed rewarding experiences (mating, ethanol exposure, or NPF circuit activation) with one of two neutral odor cues. After 24 hours, flies were tested for their odor preference; development of a preference for the odor associated with these experiences would imply that flies found the events rewarding. To test if mating is rewarding, males were exposed sequentially for 30 min to two odorants [ethyl acetate (EA) or isoamyl alcohol (IAA)], one in the absence and the other in the presence of virgin

females, and tested for odor preference 24 hours later in the absence of females. A conditioned odor preference index (CPI) for mating was calculated by averaging preference indices for reciprocally trained groups of flies. Positive CPI values indicate conditioned preference, negative values indicate aversion. Males displayed a strong preference for the mating-associated odor (Fig. 4A). We have separately shown that flies exhibit conditioned preference for an odor associated with ethanol intoxication in a similar assay (4). Together, these results indicate that both mating and ethanol intoxication, the latter of which is likely achieved in the two-choice consumption assay (5), are indeed rewarding experiences to male flies.

To test whether activation of the NPF-NPFR pathway is rewarding per se, we trained virgin males to associate artificial activation of NPF neurons with either EA or IAA. Males expressing dTRPA1 in NPF neurons (*NPf-GAL4 + UAS-dTRPA1*) and the genetic controls each carrying only one of the two transgenes were trained for three 1-hour sessions at 29°C, with dTRPA1 active, interspersed with three 1-hour rest periods at 18°C, with dTRPA1 inactive (Fig. 4B). When tested 24 hours later, males in the experimental group demonstrated strong preference for the odor associated with NPF neuron activation. The genetic controls, which did not undergo NPF neuron activation, but were exposed to the same training protocol, developed no odor preference (Fig. 4C). Other controls, which underwent NPF neuron activation but were not exposed to the training protocol, similarly developed no odor preference (fig. S6C). Thus, activation of the NPF-NPFR system is in itself rewarding to flies.

We next tested whether artificial activation of the NPF-NPFR system diminishes ethanol reward seeking. Flies were trained to associate EA or IAA with a moderately intoxicating exposure of ethanol vapor (three 10-min training sessions spaced by 1 hour) as described before (4). Wild-type flies normally show conditioned aversion to ethanol (negative CPI) when tested 30 min after training, and conditioned preference 24 hours later (4). We used this assay to compare virgin male flies that expressed dTRPA1 in NPF neurons (*NPf-GAL4 + UAS-dTRPA1*) with genetic controls that did not. Artificial activation of NPF cells, which occurs at 30°C but not 22°C, had no effect on the initial aversion (fig. S6, A and B), but abolished conditioned preference for ethanol (Fig. 4, D and E). Thus, NPF neuron activation, which is in itself rewarding to flies, interferes with the ability of flies to form a positive association between ethanol intoxication and an odor cue, which is reflected in lower ethanol consumption.

If the NPF-NPFR system were to function generally to signal the state of the *Drosophila* reward system, NPF levels should be increased by rewarding experiences other than mating, such as exposure to intoxicating levels of ethanol. To test this hypothesis, we exposed virgin males to ethanol vapor using an exposure paradigm previously shown to be rewarding (three 10-min exposures spaced by 1 hour) (4). NPF transcript levels increased 1 hour after exposure and returned to basal levels 24 hours later (Fig. 4F). Because the ethanol-induced changes in NPF levels are transient, whereas the memory of ethanol reward lasts for several days, it is possible that ethanol-induced changes in NPF levels set in motion a process, likely involving dopaminergic systems (4, 22), that modifies the fly reward system. Indeed, the activity of the NPF circuit could remain altered long after the levels of NPF had returned to baseline. Regardless of the exact mechanism, these data suggest that activity of the NPF system is regulated by at least two rewarding experiences, mating and ethanol intoxication.

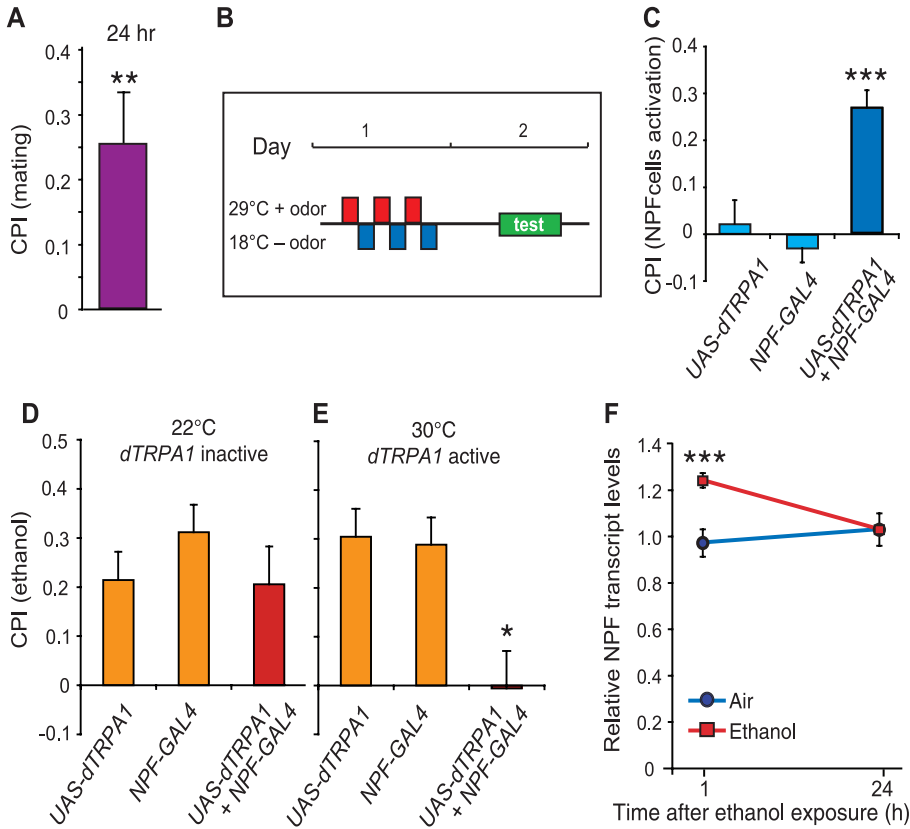


Fig. 4. Mating and NPF cell activation are rewarding and reduce ethanol reward. **(A)** Mating is rewarding to male flies. Males trained to associate an odor with mating (presence of virgin females) develop preference for that odor. P values were calculated by Wilcoxon analysis against zero. Mating against zero was $**P = 0.001$; each reciprocal group against zero was $P = 0.004$ for one odor (IAA) plus mating and $P = 0.02$ for the reciprocal odor (EA) plus mating. CPI, conditioned preference index (calculated by averaging the odor preference indexes for reciprocally trained males). **(B and C)** NPF cell activation is rewarding. Males expressing dTRPA1 in NPF neurons (NPF-GAL4 + UAS-dTRPA1) and the genetic controls carrying either transgene alone were exposed to three 1-hour training sessions at 29°C in the presence of odor [red rectangles in (B)] that were spaced by 1-hour rest periods at 18°C in the absence of odor [blue rectangles in (B)]. Testing for odor preference was performed 24 hours after training at 21°C. Experimental males, but not the genetic controls, showed preference for the odor that was associated with dTRPA1 activation in NPF neurons. Data are averages of three independent experiments. Statistical analysis was carried out by two-way ANOVA with Bonferroni post tests; comparisons are between treatment groups ($**P < 0.001$, $n = 24$). **(D and E)** Activation of NPF neurons abolishes ethanol reward. Activation of NPF neurons using dTRPA1 (NPF-GAL4 + UAS-dTRPA1) eliminated conditioned ethanol preference compared to the singly transgenic controls when tested 24 hours after training ($*P < 0.01$, one-way ANOVA with Wilcoxon/Kruskal-Wallis post-hoc tests, $n = 22$). **(F)** NPF transcript levels are induced by ethanol intoxication. Males were exposed to moderately intoxicating levels of ethanol vapor (three 10-min ethanol exposures spaced by 1 hour), collected, and frozen 1 or 24 hours later. NPF mRNA levels, measured by qPCR, were elevated 1 hour after ethanol exposure and returned to basal level after 24 hours ($**P < 0.001$ compared to air-exposed controls, Dunnett's test, $n = 3$ independent experiments with 30 males each).

NPF has been shown to influence several complex behaviors in flies, including larval intake of noxious food (23), a switch in feeding behavior (24), and responses to physical stressors (25) and ethanol (16). In addition, NPF neurons modulate the effect of satiety on sugar reward memory (22). In our paradigm, NPF appears to play a different role: Its expression is regulated by sexual experience and ethanol intoxication, and activation of NPF neurons acts as a reward signal, thereby abolishing ethanol reward and the enhanced ethanol consumption observed after

sexual deprivation. It is likely that the effects of NPF we describe here are mediated by a different set of NPF-expressing neurons than those mediating NPF's role in sugar reward memory.

Mammalian NPY has several distinct behavioral functions that are mediated by different brain regions, including roles in feeding (26, 27), anxiety, stress (28), sleep regulation (29), sexual motivation (30), and ethanol consumption (15, 31). Stressors also regulate NPY levels (18–20). In addition, injection of NPY into the nucleus accumbens of rats is rewarding (32), and NPY administration

relieves the negative affective states of drug withdrawal and depression (33, 34).

Our findings are thus not only consistent with known functions of mammalian NPY and its mode of regulation, but also provide evidence for NPF functioning as a key molecular transducer between social experience and drug reward. *Drosophila* is a useful and accessible model system in which to decipher the mechanisms by which social experiences interact with reward systems.

References and Notes

1. A. E. Kelley, K. C. Berridge, *J. Neurosci.* **22**, 3306 (2002).
2. S. E. Hyman, *Am. J. Bioeth.* **7**, 8 (2007).
3. G. F. Koob, *Neuropharmacology* **56** (suppl. 1), 18 (2009).
4. K. R. Kaun, R. Azanchi, Z. Maung, J. Hirsh, U. Heberlein, *Nat. Neurosci.* **14**, 612 (2011).
5. A. V. Devineni, U. Heberlein, *Curr. Biol.* **19**, 2126 (2009).
6. G. E. Robinson, R. D. Fernald, D. F. Clayton, *Science* **322**, 896 (2008).
7. V. Krishnan, E. J. Nestler, *Curr. Top. Behav. Neurosci.* **7**, 121 (2011).
8. S. M. McBride et al., *Neuron* **24**, 967 (1999).
9. J. E. Mehren, A. Ejima, L. C. Griffith, *Curr. Opin. Neurobiol.* **14**, 745 (2004).
10. W. W. Ja et al., *Proc. Natl. Acad. Sci. U.S.A.* **104**, 8253 (2007).
11. Materials and methods are available as supporting material on Science Online.
12. A. Ejima et al., *Curr. Biol.* **17**, 599 (2007).
13. J. C. Billeter, J. Atallah, J. J. Krupp, J. G. Millar, J. D. Levine, *Nature* **461**, 987 (2009).
14. R. S. Hewes, P. H. Taghert, *Genome Res.* **11**, 1126 (2001).
15. T. E. Thiele, D. J. Marsh, L. Ste. Marie, I. L. Bernstein, R. D. Palmiter, *Nature* **396**, 366 (1998).
16. T. Wen, C. A. Parrish, D. Xu, Q. Wu, P. Shen, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 2141 (2005).
17. A. G. Davies, J. C. Bettinger, T. R. Thiele, M. E. Judy, S. L. McIntire, *Neuron* **42**, 731 (2004).
18. A. Thorsell, K. Carlsson, R. Ekman, M. Heilig, *Neuroreport* **10**, 3003 (1999).
19. P. A. Jiménez-Vasquez, A. A. Mathé, J. D. Thomas, E. P. Riley, C. L. Ehlers, *Brain Res. Dev. Brain Res.* **131**, 149 (2001).
20. R. Sah et al., *Biol. Psychiatry* **66**, 705 (2009).
21. M. Rosenzweig et al., *Genes Dev.* **19**, 419 (2005).
22. M. J. Krashes et al., *Cell* **139**, 416 (2009).
23. Q. Wu, Z. Zhao, P. Shen, *Nat. Neurosci.* **8**, 1350 (2005).
24. Q. Wu et al., *Neuron* **39**, 147 (2003).
25. J. Xu, M. Li, P. Shen, *J. Neurosci.* **30**, 2504 (2010).
26. A. Thorsell, R. Rimondini, M. Heilig, *Neurosci. Lett.* **332**, 1 (2002).
27. T. M. Hahn, J. F. Breininger, D. G. Baskin, M. W. Schwartz, *Nat. Neurosci.* **1**, 271 (1998).
28. M. Heilig, *Neuropeptides* **38**, 213 (2004).
29. M. Dyzma, K. Z. Boudjeltia, B. Faraut, M. Kerkhofs, *Sleep Med. Rev.* **14**, 161 (2010).
30. S. P. Kalra, J. T. Clark, A. Sahu, M. G. Dube, P. S. Kalra, *Synapse* **2**, 254 (1988).
31. A. Thorsell, *Peptides* **28**, 480 (2007).
32. S. A. Josselyn, R. J. Beninger, *Pharmacol. Biochem. Behav.* **46**, 543 (1993).
33. J. P. Redrobe, Y. Dumont, R. Quirion, *Life Sci.* **71**, 2921 (2002).
34. K. A. Stogner, P. V. Holmes, *Eur. J. Pharmacol.* **387**, R9 (2000).

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as well as supplementary figures are available as supporting online material.

Supporting Online Material

www.sciencemag.org/cgi/content/full/335/6074/1351/DC1
Materials and Methods

Figs. S1 to S6
References

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SNARE Proteins: One to Fuse and Three to Keep the Nascent Fusion Pore Open

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Neurotransmitters are released through nascent fusion pores, which ordinarily dilate after bilayer fusion, preventing consistent biochemical studies. We used lipid bilayer nanodiscs as fusion partners; their rigid protein framework prevents dilation and reveals properties of the fusion pore induced by SNARE (soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor). We found that although only one SNARE per nanodisc is required for maximum rates of bilayer fusion, efficient release of content on the physiologically relevant time scale of synaptic transmission apparently requires three or more SNARE complexes (SNAREpins) and the native transmembrane domain of vesicle-associated membrane protein 2 (VAMP2). We suggest that several SNAREpins simultaneously zipper their SNARE transmembrane helices within the freshly fused bilayers provide a radial force that prevents the nascent pore from resealing during synchronous neurotransmitter release.

Efficient synaptic transmission requires the fast release of neurotransmitters from synaptic vesicles that fuse with the presynaptic plasma membrane upon entry of calcium ions (1). Membrane fusion necessarily implies a fusion pore that opens between the vesicle and its partner membrane at the instant of fusion. The conductance properties of such nascent fusion pores suggest that their typical diameters are in the range of ~2 nm, although considerable variability exists (2–5). Neurotransmitter is released from synaptic vesicles [diameter ~40 nm (6, 7)] by diffusion through the nascent pore in the first 100 to 200 μ s, even before appreciable dilation of the pore occurs (4). The transient and variable nature of the fusion pore has severely limited biochemical and physical chemical studies.

We suggest that nanodiscs (8–11) provide an ideal model for such studies because the small amount of disc lipid will suffice to allow pores to open but not expand (Fig. 1 and Fig. 2A) beyond their nascent, physiologically relevant state for neurotransmitter release. Nanodiscs are synthetic lipoprotein particles that contain a small piece of circular lipid bilayer (up to ~17 nm in diameter) wrapped by two copies of membrane scaffold protein (MSP) derived from apolipoprotein A1. In the system we describe here, nanodiscs contain the synaptic vesicle SNARE (v-SNARE) VAMP2

and small unilamellar vesicles [diameter 30 to 60 nm (12)] contain the synaptic target membrane SNARE (t-SNARE) complex of syntaxin 1 and SNAP25. SNAREs are the core machinery for this and other cellular membrane fusion processes (12–14). They assemble between bilayers as a four-helix bundle (15) that imparts sufficient force to cause bilayer fusion (16).

After reconstitution (supplementary text), the nanodiscs containing VAMP2 (v-discs) were separated by gel filtration (Fig. 1, A to C). Each disc contained about 400 lipid molecules wrapped by two MSPs. With a starting VAMP2/MSP ratio of 6:2, we recovered ~7 VAMP2 copies per disc on average after removing VAMP2-free discs. Electron microscopy of v-discs confirmed an average diameter of 16 ± 2 nm (Fig. 1D). Not surprisingly, single VAMP2 proteins on these discs could not be readily distinguished because of their small size and flexible structure. However, addition of the soluble t-SNARE (a complex of syntaxin H3 cytosolic domain and SNAP25/N/C helical domains) formed rodlike SNARE complexes that were seen to protrude from the nanodiscs (Fig. 1D). This confirms that VAMP2 on nanodiscs can form SNARE complexes.

We used a well-established lipid mixing assay (17) to test whether v-discs can fuse with t-vesicles. Nitro-2-1,3-benzoxadiazol-4-yl-phosphatidylethanolamine (NBD-PE) and rhodamine-PE were included in the v-discs (1.5 mol % each). This surface concentration of rhodamine effectively quenches the NBD fluorescence. However, when a nanodisc fuses with a liposome, NBD fluorescence will greatly increase because of the substantial (>50-fold) lipid dilution as the disc lipid mixes with the massive ex-

cess of vesicle lipid, as we observed (Fig. 2B). Little or no increase of NBD signal was observed in control experiments. The slow lipid mixing between nanodiscs and vesicles was limited by the rate of docking (initial SNARE assembly) and not by the rate of fusion (fig. S1), as is the case for vesicle-vesicle fusion systems (18). A similar fusion process was observed when the SNAREs were placed in the opposite topology, with v-SNAREs in the vesicle and t-SNAREs in the nanodisc (fig. S2).

To monitor efflux of content via fusion pores that necessarily form at least transiently during the fusion process, we encapsulated calcium (50 mM) in the liposomes, which were then incubated with v-discs in a medium containing a calcium-activated fluorophore, Mag-Fluo-4 (2 μ M, K_d for calcium = 22 μ M; Invitrogen). When pores open, calcium diffuses through the pores into the exterior buffer, inducing a fluorescence signal. The results (Fig. 2C) clearly show that calcium was released in a SNARE-specific manner. To ascertain that this efflux was due to diffusion through a pore, rather than transient lysis or leakage of the vesicle during fusion, we tested the rate of release of vesicle cargos of different sizes—specifically, the calcium chelator EDTA (Stokes radius, ~0.4 nm) and EGTA (Stokes radius, ~0.5 nm). EDTA release from liposomes was faster than EGTA release from liposomes by a factor of 2.3 (fig. S3). From these data, a pore size of ~2 nm can be calculated (supplementary text), similar in size to the nascent fusion pore size calculated from electrophysiological measurements (3).

When vesicles fuse to target membranes, the fusion pore eventually expands and the vesicle is incorporated into the target membrane. With nanodiscs, however, the pore cannot appreciably expand beyond its nascent diameter of ~2 nm, so the only means available to reduce membrane stress (resulting from the extreme curvature inherent in a small pore) is for the pore to eventually reseal. To confirm the prediction that nanodisc-vesicle pores reseal, we introduced dithionite (5 mM) into samples 40 min after beginning the fusion assay. Dithionite quenches all externally accessible NBD (19), including NBD on both faces of unreacted nanodiscs and NBD-PE that had diffused into the outer leaflets of liposomes via hemifusion or full fusion. Dithionite is also small enough (Stokes radius, 0.2 to 0.3 nm) to readily diffuse through any 2-nm fusion pores that may remain open and quench the NBD signal on the inner leaflets in the case that the pore remains open, but will not gain access to the interior if the pore has (as predicted) closed off. We observed that some of the NBD dye remained protected against dithionite, and only

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after the fusion reaction (Fig. 2D and fig. S4). No NBD protection was found for the negative controls. Thus, there are some fusion events that correspond to full fusion between the nanodisc and the liposome, in which a pore must have opened and then resealed. Reverse experiments where NBD-PE was initially only on the t-liposomes confirmed that essentially no pores remained open after full fusion (fig. S5). The dithionite data indicate that ~ 50% of all fusion events entailed full fusion with subsequent resealing of the pore (see supplementary text). The ~50% balance of SNARE complex-dependent events can be accounted for by events resembling hemifusion in which no pore opens and only the outer

leaflets are shared between the liposome and nanodisc. In all cases, the nanodisc remained attached to the liposomes after the pore resealed (fig. S6, A to D), consistent with the idea that after full fusion, the pore reseals to a hemifusion-like state in which a stalk of lipid bilayer permanently connects the outer leaflet of the vesicle to what had been the SNARE complex-containing leaflet of the nanodisc (fig. S6F). After resealing, VAMP2 is fully resistant to toxin cleavage, which suggests that it is in cis-SNARE complexes (fig. S6, E and F).
The number of VAMP2 copies per disc can be controlled by adjusting the input VAMP2/MSP ratio. With increasing VAMP2/MSP ratios during

assembly of nanodiscs, the v-disc products eluted progressively earlier on gel filtration columns (Superdex 200), consistent with increasing size and more VAMP2 being inserted into each disc (Fig. 3A). To test how the number of SNAREpins affects fusion, we purified seven sets of nanodiscs containing, respectively, an average of 1.2 (ND1), 2.2 (ND2), 3.15 (ND3), 4.3 (ND4), 5.5 (ND5), 7.4 (ND7), and 9.3 (ND9) copies of VAMP2 after VAMP2-free nanodiscs were removed by affinity purification (Fig. 3B and fig. S7). VAMP2 and MSP were mixed in these preparations in different proportions, and the VAMP2/MSP ratios in the final isolated nanodiscs used in the fusion experiments were established by three independent

Fig. 1. (A) Cartoon showing the v-disc model. The nanodisc is a small piece of lipid bilayer wrapped by two MSPs (blue). VAMP2 (green) can insert into a nanodisc to form a v-disc (11). The lipid head groups are shown as gray spheres. **(B)** Elution profile of nanodisc or v-disc on Superdex 200 10/300 GL column. Embedment of VAMP2 results in the earlier elute volume of a v-disc (red major peak) relative to that of a VAMP2-free nanodisc (black peak). By gel filtration, the 6xHis-SUMO tag (cleaved from VAMP2 by SUMO-protease, the red minor peak) can also be removed. **(C)** SDS–polyacrylamide gel electrophoresis (PAGE) gel stained with Coomassie Brilliant Blue showing the input and final nanodisc products after gel filtration. **(D)** V-disc samples were analyzed in an FEI Tecnai-12 electron microscope. Left panel: V-discs show regular “disc” shapes; VAMP2 protein can hardly be seen because of small protein size and flexible structure. Right panel: When soluble syntaxin 1A H3 domain and SNAP-25N/C domain were co-incubated with v-disc, they form SNARE complexes that can be seen as rodlike structures (red) protruding from two sides of the v-disc (green).

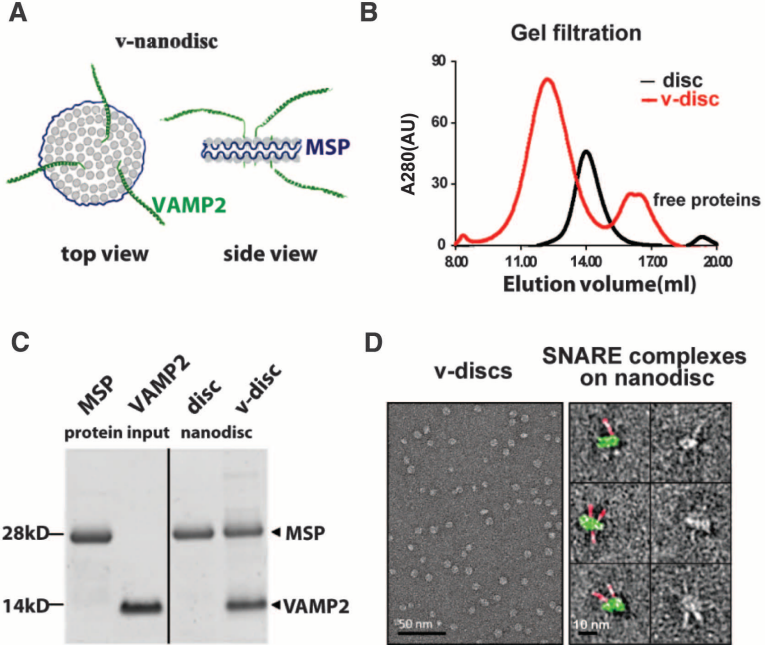
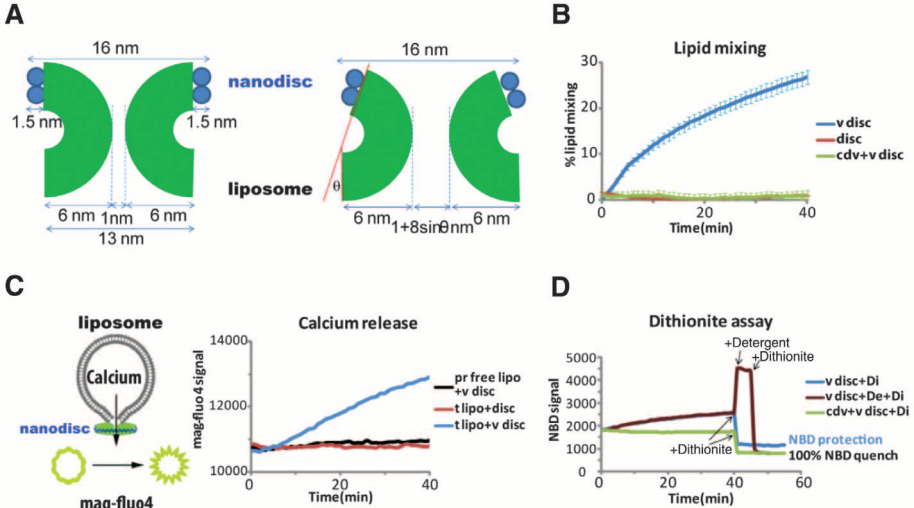


Fig. 2. (A) Schematics showing how the fusion pore can be envisioned. The diameter of the nanodisc is 16 nm. Lipids that naturally form flat surfaces will favor structures that have a zero net curvature (when neglecting the Gaussian curvature). Hence, in neck-like structures, the negative curvatures (shown here as perpendicular to the pore) are of the same order; likewise, for saddle-like structures, the positive (pore) curvatures are of the same order. A 4-nm pore would correspond to a 6-nm curvature for the exterior of the bilayer. This is approximately what is represented here. With no stress, a 1-nm pore can form. With a reasonable amount of stress ($\theta = 20^\circ$), a 4-nm pore diameter would result. **(B)** Lipid mixing is SNARE-specific. V-discs exchanged lipids with t-liposomes (blue). Discs without VAMP2 do not fuse with t-liposomes (red); cytoplasmic domain of VAMP2 (CDV), which titrates the free t-SNARE, also blocks the fusion (green). **(C)** Calcium release is SNARE-specific. Calcium (50 mM) is encapsulated into t-liposome; during the liposome-nanodisc fusion, the pore opens, calcium is released from the liposome to the exterior buffer, and the Mag-Fluo-4 signal is enhanced. An increasing Mag-Fluo-4 signal indicates that calcium is continuously released during the fusion (blue); limited calcium release is observed under nonfusogenic conditions (red and black). **(D)** Dithionite assay



showed some NBD protection after 40 min of fusion (blue). To completely quench all NBD signal, detergent (De) was first added to disrupt the liposomes, followed by dithionite (Di) to obtain 100% quench (brown). With CDV to block the fusion, no NBD protection was observed after dithionite treatment (green).

methods: Coomassie Blue–based protein determinations (Fig. 3B), quantitative Western blotting (fig. S7), and counting of the discrete photobleaching steps of single nanodisc particles containing fluorescently labeled VAMP2 (fig. S13). The three methods agreed very closely [within $\pm 3\%$ (SD/mean $\times 100$) for ND1 and ND2 in particular; see table S2].

Remarkably, all seven samples drove lipid mixing at the same rate (Fig. 3C), which implies that a single v-SNARE per nanodisc yields the maximum rate of membrane fusion. Furthermore, the dithionite protection assay shows that for any VAMP2 copy number, the percentage of lipid-mixing events corresponding to full fusion remains unchanged at $45 \pm 7\%$ (SD; fig. S8). Together, these two results clearly establish that a single SNAREpin is sufficient to drive complete membrane fusion.

The pores forming in our nanodisc-liposome system are transient, eventually resealing of their own accord (fig. S5). Because our lipid-mixing experiments result in full membrane fusion (i.e., full exchange of lipid), we can set a lower limit for

how long these pores must have remained open. In order for all of the fluorescent phospholipid to equilibrate between the nanodisc and the liposome, the pore must remain open for $\sim 10 \mu\text{s}$ (it takes $\sim 10 \mu\text{s}$ for a phospholipid with a diffusion coefficient of $5 \mu\text{m}^2/\text{s}$ to cover 50 nm^2 , half of a nanodisc embedded bilayer, and reach the fusion pore). In contrast, simple considerations and *in vivo* observations of neurotransmitter release from synaptic vesicles suggest that a much longer time ($\sim 100 \mu\text{s}$) is required for full efflux of the content with similar size as neurotransmitter from $\sim 40\text{-nm}$ liposomes or synaptic vesicles (4, 20–22).

Do the fusion pores between nanodiscs and vesicles remain open long enough for such cargo to efflux? The answer (Fig. 3D) is that they do when several or more SNARE complexes can form, but not when only a single SNAREpin is available. In contrast to the rate of lipid mixing, maximum cargo efflux decreased precipitously from 12% (ND9 and ND7) to less than 2% (ND1 and ND2) as the number of VAMP2s per disc decreased from ~ 9 to ~ 1 . Thus, even though the rate and frequency of full membrane fusion events

do not depend on the number of VAMP2 molecules per disc, the efficiency of cargo release is highly sensitive to SNAREpin number, increasing markedly as the number of SNAREpins increases above two per disc.

At very low SNARE numbers (i.e., ND1 or ND2), the pore opens only long enough to exchange lipid, and thus only a small fraction of the content is released. The amount of release increases starting with ND3 discs and reaches a maximum with ND7 discs, which reconstituted with about 7 VAMP2 proteins total or about 3.5 per nanodisc face (table S2); this finding suggests that maximum efflux requires 3 or 4 VAMP2 proteins. The simplest model is that a limited content release occurs when one SNAREpin is engaged, whereas a sudden increase in content release occurs above a threshold when enough SNAREpins are involved (fig. S12C). In the nanodisc system, that critical number is achieved when any one side of the nanodisc has at least the minimum necessary number of SNAREs. Indeed, if we assume that the VAMP2 distributes randomly between the two sides of each disc (fig. S12B), the calcium release across the whole of the titration fits well to such a “cooperative” model and describes the threshold number of SNAREpins for efficient content release as ~ 3 (fig. S12C), which is consistent with *in vivo* observations.

The role of the SNARE transmembrane domains (TMDs) in fusion has been unclear. Membrane anchorage of the assembling cytosolic domains of VAMP2 and syntaxin is needed, and when this is provided by membrane-spanning lipids, fusion still occurs (17). In absolute contrast, point mutations in the syntaxin 1 TMD reduce the amplitude of the foot signal in electrophysiology (23), and deletion in the VAMP2 TMD significantly reduces neurotransmitter release (24). This implies a role for the TMDs either in the opening of the nascent physiological fusion pore, or in maintaining it open for the $\sim 100 \mu\text{s}$ needed for transmitter efflux, or both. Because fusion pores must open (at least transiently) when lipid anchors mediate fusion, the simplest possibility is that the TMDs somehow keep the fusion pore from resealing when transmitter is exiting and the pore has not begun to appreciably expand. In this connection, it is noteworthy that the VAMP2 and syntaxin TMDs extend as a two-helix bundle through the entire span of the membrane (25). This raises the possibility that force resulting from the terminal zippering of SNARE TMDs within the bilayer could provide a source of energy to tip the balance against resealing in the nascent fusion pore.

To test this hypothesis, we used three chimeric VAMP2 proteins in which the VAMP2 TMD was replaced by (i) a dioleoyl phospholipid that spans only one monolayer (C18), (ii) a long C45 isoprenoid that can span the lipid bilayer (C45), or (iii) a non-SNARE TMD from platelet-derived growth factor receptor (PDGFR) (Fig. 4A). As with the wild-type VAMP2, seven samples of PDGFR-VAMP2-discs (characterized in fig. S9)

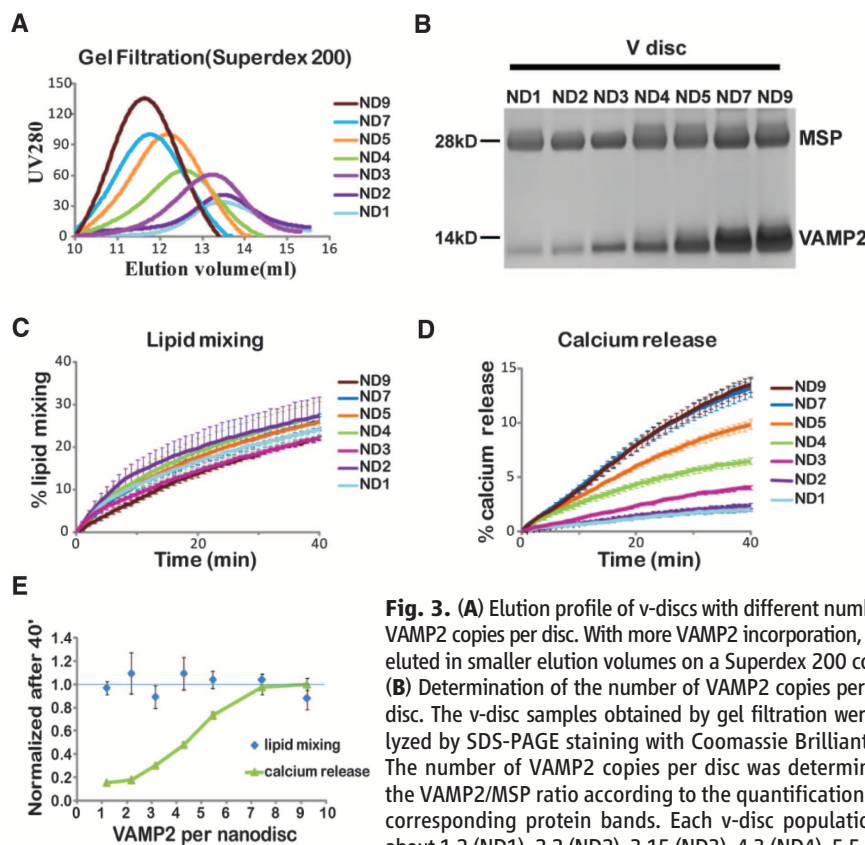


Fig. 3. (A) Elution profile of v-discs with different numbers of VAMP2 copies per disc. With more VAMP2 incorporation, v-discs eluted in smaller elution volumes on a Superdex 200 column. (B) Determination of the number of VAMP2 copies per nanodisc. The v-disc samples obtained by gel filtration were analyzed by SDS-PAGE staining with Coomassie Brilliant Blue. The number of VAMP2 copies per disc was determined by the VAMP2/MSP ratio according to the quantification of the corresponding protein bands. Each v-disc population has about 1.2 (ND1), 2.2 (ND2), 3.15 (ND3), 4.3 (ND4), 5.5 (ND5), 7.4 (ND7), and 9.3 (ND9) copies of VAMP2 per disc on average.

(C) Lipid mixing assays demonstrate that discs with varying VAMP2 copy numbers are equally efficient in fusing with calcium-encapsulated t-liposomes. (D) Calcium release assay shows different kinetics when v-discs with different copy numbers of VAMP2 fuse with t-liposomes. Calcium release is gradually increased with higher copy numbers of VAMP2 inserted into the nanodiscs. The error bars are SEM. (E) The endpoint values after a 40-min fusion reaction are presented for both lipid mixing (blue) and calcium release (green). Lipid mixing, normalized by the average endpoint value, does not vary significantly with the number of VAMP2 copies. Calcium release, normalized by the value for ND9, varies as a sigmoid with an inflection point at ~ 5 VAMP2 copies per nanodisc.

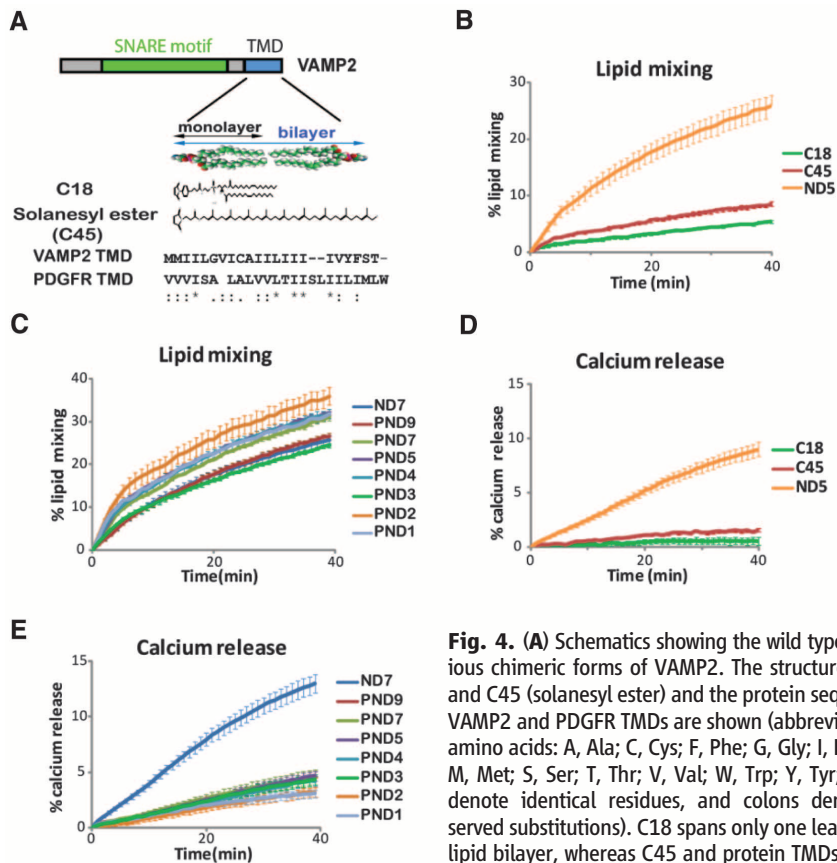


Fig. 4. (A) Schematics showing the wild type and various chimeric forms of VAMP2. The structures of C18 and C45 (solanesyl ester) and the protein sequences of VAMP2 and PDGFR TMDs are shown (abbreviations for amino acids: A, Ala; C, Cys; F, Phe; G, Gly; I, Ile; L, Leu; M, Met; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; asterisks denote identical residues, and colons denote conserved substitutions). C18 spans only one leaflet of the lipid bilayer, whereas C45 and protein TMDs cross the lipid bilayer. (B) Lipid mixing assay of t-liposome with

v-disc prepared with wild-type VAMP2 (ND5) or CDV with C18 or C45. The lipid anchor, either C18 or C45, shows compromised fusion efficiency relative to wild-type VAMP2 with native TMD. (C) Lipid mixing assay shows that the TMDs of VAMP2 and PDGFR have similar fusion kinetics, which suggests that these two TMDs are equivalent in lipid mixing. (D) Calcium release assay shows substantially reduced release when protein TMD is replaced by lipid anchor, either C18 or C45. (E) Calcium release assay reveals that VAMP2 TMD is more efficient than PDGFR TMD for content release. The error bars are SEM.

were prepared: PND9, PND7, PND5, PND4, PND3, PND2, and PND1, respectively containing an average of 9.0, 7.2, 5.7, 4.4, 3.75, 2.7, and 1.2 copies of PDGFR-VAMP2. Lipid anchors (C18 and C45, both with an average of ~5 copies per nanodisc) were less efficient for fusion than the VAMP2 TMD by a factor of 5 to 10 (Fig. 4B), whereas the PDGFR TMD fused at the same rate as the native VAMP2 TMD at all numbers of VAMP2s per nanodisc (Fig. 4C). With C18 VAMP2, the fluorescence signal collapsed back to the background level after dithionite injection (fig. S10), which suggests that only the outer leaflets are shared (i.e., hemifusion). By contrast, C45 and PDGFR TMDs achieved full fusion with essentially the same ~50% efficiency as the native VAMP2 TMD. This confirms that a membrane-spanning domain (either lipid or protein) is required to achieve full fusion and also demonstrates that fusion pores open when only the cytosolic domains of the SNARE complex have zippered. Thus, bilayer fusion and the concomitant opening of the nascent pore do not require assembly of the syntaxin and VAMP2 TMDs into the bilayer-spanning helical bundle.

To establish the lifetime of the open pore in these artificial fusion events, we used the calcium release assay. By contrast to lipid mixing, none of the nonnative VAMP2 TMDs efficiently released cargo (Fig. 4, D and E). These experiments show that the native VAMP2 TMD is specifically required for efficient release of vesicle content after the pore opens, which it allows by virtue of lowering the rate of resealing of the nascent fusion pore.

In the nanodisc system, the extent of content release can only be determined by the total amount of time the pore is open before permanently resealing. Should the pore reseal within ~100 μ s (as calculated from diffusion constants and vesicle and pore diameter), only a commensurate fraction of the cargo will exit even though the lipids in the disc and vesicle bilayers will have fully mixed. These considerations are fundamental for understanding the different requirements for the number of SNAREs and their TMDs for vesicle fusion and content release, as revealed by our nanodisc experiments. Specifically, our results suggest that although a single SNARE complex suffices to open a fusion pore between nanodisc

and vesicle, this pore is too short-lived to allow much transmitter to exit before the pore closes. Only when there are several or more SNAREpins assembling at the same pore does it remain open long enough for effective transmitter release. Even if there are enough SNAREs, it appears that the pore is short-lived unless the TMDs of VAMP2 and syntaxin can zipper in the bilayer to keep the pore open in one way or another, perhaps by pushing outward radially as their TMDs zipper within the bilayer. A single zippering SNAREpin could not do this, which explains why it would necessarily be ineffective. But it is easy to see that three or more SNAREpins pushing away from each other radially could channel the energy of trans-bilayer zippering to keep the nascent pore open. In this speculative model, the restraining force against resealing may only last for as long as it takes the TMDs to zipper. That length of time is likely to be much more than 100 μ s, based on the maximum speed for folding of a two-helix coil (26–28) and considering the higher viscosity of hydrocarbon relative to water (29); this would be more than enough time to allow complete neurotransmitter release.

A previous study showing that a single SNARE complex could mediate vesicle-vesicle fusion also measured content mixing (30). Interestingly, normalizing the published data for SNARE-free vesicles (see supplementary text and table S1) reveals that here, too, content mixing is greatly reduced relative to bilayer fusion with one SNARE complex per vesicle. Vesicle-vesicle fusion is inherently a poor model of neurotransmitter release through a nascent fusion pore because content mixing occurs not only through a nascent pore (as in nanodiscs and at the synapse) but also subsequently as the vesicles more slowly complete their fusion. Yet despite these limitations, indications of the mechanism we have uncovered can still be found.

These findings provide a simple mechanistic basis for understanding published data that had seemed to be contradictory. Titrations of dominant-interfering SNARE mutants in permeabilized or intact neurosecretory cells suggest a minimum requirement for three SNAREpins to open a fusion pore sufficient for neurotransmitter efflux (31, 32). A quantitative analysis of titrations of botulinum toxin A in relation to cleavage of SNAP25 has been interpreted to mean that a minimum of 10 to 15 SNAP25 molecules are required (33), but this analysis assumes that all SNAP25 is present in SNARE complexes and must be considered an upper limit only. By contrast, an elegant single-particle, single-molecule analysis combined with vesicle fusion reactions clearly established that a single SNAREpin was present in many fused vesicles (30). We can now see that all of these results emerge from a single underlying mechanism in which the dynamics of the nascent fusion pore are determined by the number of SNAREpins involved. Synaptic vesicles have ~70 copies of the v-SNARE VAMP2 (6) and the active zone is rich in t-SNAREs (34),

ensuring that multiple SNAREpins are always available to keep the pore open and let transmitter out as rapidly as possible.

References and Notes

1. T. C. Südhof, *Annu. Rev. Neurosci.* **27**, 509 (2004).
2. A. Albillos *et al.*, *Nature* **389**, 509 (1997).
3. L. J. Breckenridge, W. Almers, *Nature* **328**, 814 (1987).
4. D. Bruns, R. Jahn, *Nature* **377**, 62 (1995).
5. Q. Fang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 15388 (2008).
6. S. Takamori *et al.*, *Cell* **127**, 831 (2006).
7. Y. Hu, L. Qu, T. Schikorski, *Synapse* **62**, 953 (2008).
8. T. K. Ritchie *et al.*, *Methods Enzymol.* **464**, 211 (2009).
9. J. Frauenfeld *et al.*, *Nat. Struct. Mol. Biol.* **18**, 614 (2011).
10. H. Katayama *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **107**, 3453 (2010).
11. K. D. Brewer, W. Li, B. E. Horne, J. Rizo, *Proc. Natl. Acad. Sci. U.S.A.* **108**, 12723 (2011).
12. T. Weber *et al.*, *Cell* **92**, 759 (1998).
13. C. Hu *et al.*, *Science* **300**, 1745 (2003).
14. T. Söllner *et al.*, *Nature* **362**, 318 (1993).
15. R. B. Sutton, D. Fasshauer, R. Jahn, A. T. Brunger, *Nature* **395**, 347 (1998).
16. F. Li *et al.*, *Nat. Struct. Mol. Biol.* **14**, 890 (2007).
17. J. A. McNew *et al.*, *J. Cell Biol.* **150**, 105 (2000).
18. E. A. Smith, J. C. Weisshaar, *Biophys. J.* **100**, 2141 (2011).
19. Y. Xu, F. Zhang, Z. Su, J. A. McNew, Y. K. Shin, *Nat. Struct. Mol. Biol.* **12**, 417 (2005).
20. R. G. Staal, E. V. Mosharov, D. Sulzer, *Nat. Neurosci.* **7**, 341 (2004).
21. E. N. Pothos, V. Davila, D. Sulzer, *J. Neurosci.* **18**, 4106 (1998).
22. R. Khanin, H. Parnas, L. Segel, *Biophys. J.* **67**, 966 (1994).
23. X. Han, C. T. Wang, J. Bai, E. R. Chapman, M. B. Jackson, *Science* **304**, 289 (2004).
24. E. Fdez, M. Martínez-Salvador, M. Beard, P. Woodman, S. Hilfiker, *J. Cell Sci.* **123**, 2473 (2010).
25. A. Stein, G. Weber, M. C. Wahl, R. Jahn, *Nature* **460**, 525 (2009).
26. J. Kubelka, J. Hofrichter, W. A. Eaton, *Curr. Opin. Struct. Biol.* **14**, 76 (2004).
27. W. Y. Yang, M. Gruebele, *Nature* **423**, 193 (2003).
28. K. A. Dill, S. B. Ozkan, T. R. Weikl, J. D. Chodera, V. A. Voelz, *Curr. Opin. Struct. Biol.* **17**, 342 (2007).
29. E. Karatekin, O. Sandre, F. Brochard-Wyart, *Polym. Int.* **52**, 486 (2003).
30. G. van den Bogaart *et al.*, *Nat. Struct. Mol. Biol.* **17**, 358 (2010).
31. Y. Hua, R. H. Scheller, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 8065 (2001).
32. R. Mohrmann, H. de Wit, M. Verhage, E. Neher, J. B. Sørensen, *Science* **330**, 502 (2010).
33. C. Montecucco, G. Schiavo, S. Pantano, *Trends Biochem. Sci.* **30**, 367 (2005).
34. T. Lang *et al.*, *EMBO J.* **20**, 2202 (2001).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/335/6074/1355/DC1
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SOM Text
Figs. S1 to S13
Tables S1 and S2
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ER Cargo Properties Specify a Requirement for COPII Coat Rigidity Mediated by Sec13p

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Eukaryotic secretory proteins exit the endoplasmic reticulum (ER) via transport vesicles generated by the essential coat protein complex II (COPII) proteins. The outer coat complex, Sec13-Sec31, forms a scaffold that is thought to enforce curvature. By exploiting yeast bypass-of-sec-thirteen (*bst*) mutants, where Sec13p is dispensable, we probed the relationship between a compromised COPII coat and the cellular context in which it could still function. Genetic and biochemical analyses suggested that Sec13p was required to generate vesicles from membranes that contained asymmetrically distributed cargoes that were likely to confer opposing curvature. Thus, Sec13p may rigidify the COPII cage and increase its membrane-bending capacity; this function could be bypassed when a *bst* mutation renders the membrane more deformable.

Hierarchical assembly of the COPII coat on the cytosolic face of the endoplasmic reticulum (ER) membrane couples cargo selection with membrane deformation to generate functional transport vesicles (1). All members of the COPII coat are thought to contribute to generation of membrane curvature (2, 3); however, the precise mechanism of membrane deformation by the COPII coat remains unclear (4). We sought to probe this process by dissecting the molecular function of the outer coat complex, composed of Sec13 and Sec31, which is thought to drive membrane curvature by polymerization into a latticelike spherical structure (3). Sec13 is unique among COPII proteins in performing

multiple functions, driven by structurally analogous interactions with distinct partners, including its canonical partner Sec31 (5), the nucleoporin Nup145 (6), and the COPII scaffold Sec16 (7). Given the pleiotropic functions of Sec13 and the essential nature of COPII-mediated traffic, it is surprising that yeast Sec13p is dispensable in the context of bypass-of-sec-thirteen (*bst*) mutations (8, 9). We sought to exploit this phenotype to probe vesicle formation in the context of a compromised coat complex.

The COPII “cage” self-assembles from rod-shaped Sec13-Sec31 edge elements, four of which come together at vertex regions (3). Sec31 is thought to drive assembly: The edge element is formed by stable dimerization at the α -solenoid ancestral coat element (ACE) domain; four edge elements come together at cage vertices via N-terminal β propellers (5). Sec13 lies sandwiched between the ACE and β -propeller domains, forming a six-bladed β propeller that is comple-

mented by an additional β blade formed by the domain insertion motif (DIM) of Sec31 (Fig. 1A). We first demonstrated that the essential function of yeast Sec13p was in the COPII coat by restricting its interaction to Sec31p (10): An in-frame fusion where Sec13p was inserted immediately downstream of the Sec31p DIM complemented both *sec13Δ* and *sec31Δ* strains, whereas a fusion containing the Sec13p structural homolog, Seh1p, was unable to support viability (Fig. 1B) despite being functional in a *bstΔ* background (fig. S1). Furthermore, uncoupling Sec13p from the COPII coat by mutation of the Sec31p DIM, either by point mutations (*sec31-DK*) or by complete replacement with a 13 amino acid stretch of Gly-Ser repeats (*sec31-GS₁₃*), failed to support viability except in the context of an additional *bst1Δ* mutation (Fig. 1C), effectively mimicking a *sec13Δ* mutation. We investigated whether Sec31p could engage with the COPII coat independent of Sec13p. Indeed, Sec31p, expressed and purified from insect cells to preclude copurification of Sec13p, was efficiently recruited to synthetic liposomes in the presence of the inner COPII coat, Sar1p-Sec23p-Sec24p (Fig. 1D). Furthermore, the Sec31p DIM mutants also assembled with the inner coat but were unable to recruit Sec13p (Fig. 1D). Finally, we used an in vitro vesicle formation assay (11) to confirm that Sec31p was sufficient to generate COPII vesicles in the absence of Sec13p, albeit with reduced efficiency (Fig. 1E).

If Sec13p can generate COPII vesicles on its own, what is the molecular function of Sec13p and what are the in vivo conditions created by the *bst* mutations that permit Sec31p to function in its absence? We used synthetic genetic array technology (12) to exhaustively survey the yeast genome for *BST* genes. Two query mutations—*sec31-GS₁₃* and *sec13Δ*—were introduced into the yeast deletion collection, and haploid double mutants were scored for viability (Fig. 2A and fig. S2). Three comprehensive screens yielded

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Fig. 1. Sec31p mutants mimic the loss of Sec13p. **(A)** Schematic and structural representations of Sec31p (green) and Sec13p (orange) showing the Sec31p DIM (blue), β -propeller (light green), and ACE (dark green) domains. Residues (W386D and A387K) altered in the Sec31p-DK mutant are indicated, and numbering denotes Sec31p residues. **(B)** Schematic of the Sec31-Sec13p fusion, which was introduced into *sec31 Δ* and *sec13 Δ* strains as indicated and grown on media containing 5-fluoroorotic acid (5-FOA) to counterselect for the wild-type plasmid-borne copies of *SEC13* or *SEC31*. **(C)** A *sec31 Δ* strain containing a plasmid-borne *sec31-GS₁₃* and *sec31-DK* mutant as the sole copy of *SEC31* was viable only in *bst* mutant backgrounds. **(D)** Synthetic liposomes were incubated with the COPII coat proteins and the guanine nucleotides indicated, and coat assembly was assessed by liposome flotation and SDS–polyacrylamide gel electrophoresis. Numbered lanes contain liposome-bound proteins. Asterisks denote a degradation product of Sec31p. **(E)** Microsomal membranes (T indicates 10% of total input membranes) purified from *bst1 Δ* cells were incubated with Sar1p and Sec23/24p; supplemented with Sec13p, Sec31p, and guanine nucleotides as indicated; and vesicles released were purified and analyzed by immunoblotting against cargo proteins Sec22p and Erv46p.

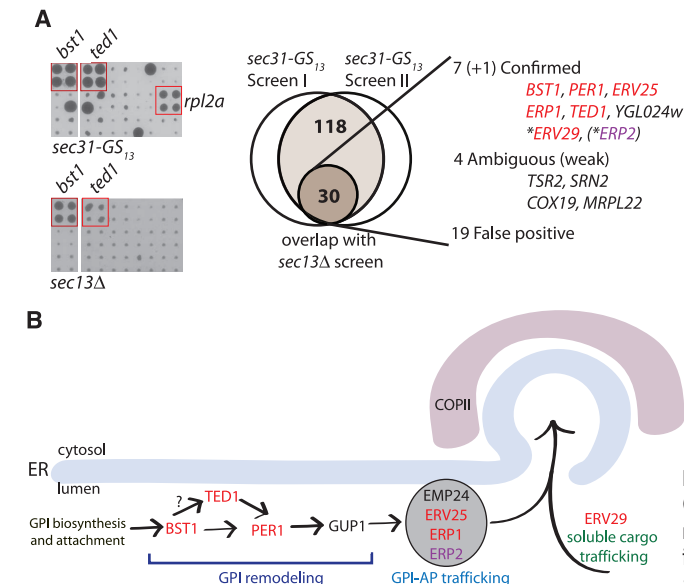
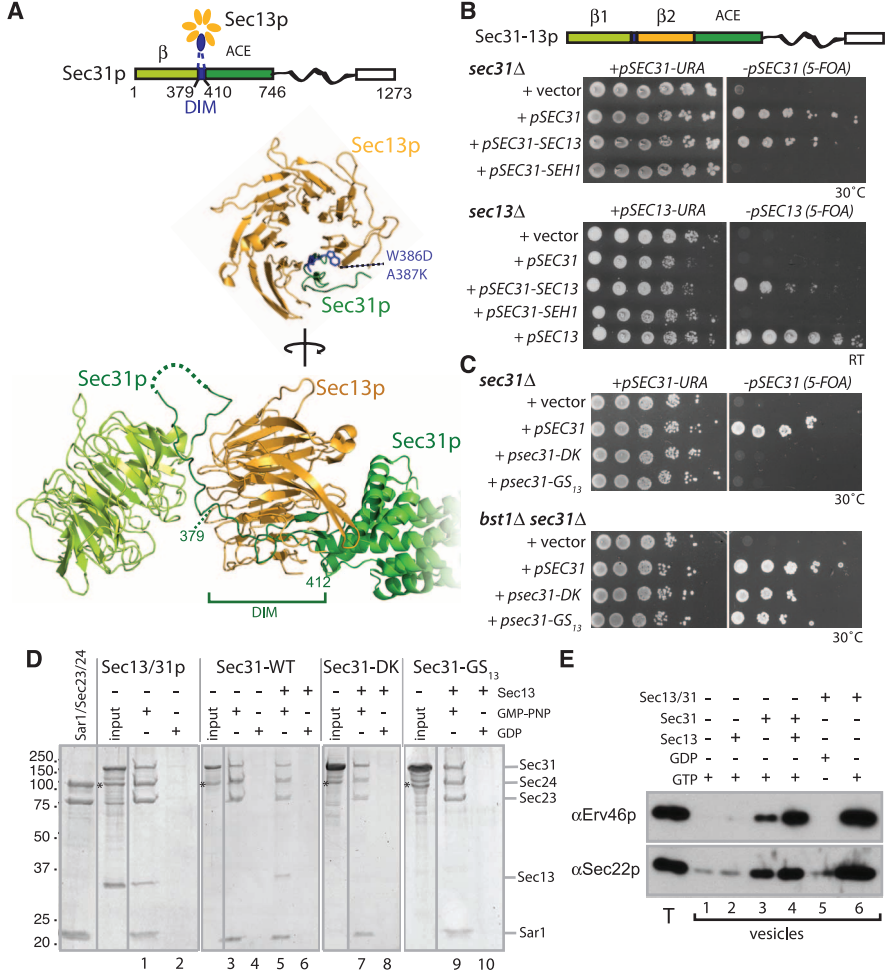


Fig. 2. Strong *bst* mutants affect export of GPI-anchored proteins from the ER. **(A)** Synthetic genetic array screen summary showing examples of hits (quaduplicate spots are highlighted) on 5-FOA plates (left), screen outcomes from two independent *sec31-GS₁₃* screens and overlap with *sec13 Δ* screen hits (middle), and a list of overlap hits (right) that were further confirmed by direct crosses with *sec13 Δ* or *sec13 Δ erv1 Δ* (*) strains. *ERP2* is functionally related to other hits (highlighted in red) but was identified only in *sec31-GS₁₃* screens. **(B)** Most confirmed *BST* genes act in GPI-AP remodeling and trafficking pathways. *EMP24* and *GUP1*, although not identified in the screens, conferred *bst* phenotypes upon direct testing. **(C)** Growth phenotypes of *bst sec13 Δ* strains generated by direct crosses on 5-FOA at 25°C. The phenotypes of *gup1 Δ sec13 Δ* and *per1 Δ sec13 Δ* depend on the strain background (*). **(D)** Maturation (trafficking) of the GPI-AP Gas1p in *p24* and *erv29 Δ* mutants, as revealed by pulse-chase experiments. Maturation of a soluble protein, CPY, is shown for comparison. p, precursor (ER) forms; m, mature (post-Golgi) forms.

118 preliminary hits, 30 of which were identified using both query strains (Fig. 2A and table S1). Many of these overlapping hits were false positives, and a few were ambiguous because their phenotypes were too weak to be conclusively confirmed (Fig. 2A and fig. S3). Our screens thus yielded seven core *bst* mutants, and by direct testing we identified three additional functionally related hits (Fig. 2, A to C).

The majority of *BST* gene products fell into two functional categories (Fig. 2B): Bst1p, Ted1p, Per1p, and Gup1p are ER enzymes that catalyze remodeling of glycosylphosphatidylinositol (GPI) anchors after proteins have been covalently attached (13, 14); Emp24p, Erv25p, Erp1p, and Erp2p are members of the p24 family and form a physical complex that shuttles between the ER and the Golgi apparatus and is required for efficient ER export of GPI-anchored proteins (GPI-APs) (9, 15, 16). Maturation of the GPI-AP, Gas1p, was defective in each of these two classes of *bst* mutants (Fig. 2D) (17); in the case of the p24 mutants, the strength of the GPI-AP defect mirrored the strength of the *bst*

phenotype (Fig. 2, C and D, and fig. S4, A and B). Two final core *BSTs* were *YGL024w*, a dubious open reading frame (fig. S5), and *ERV29*, an ER export receptor for soluble cargo proteins (18, 19). *ERV29* deletion conferred a weak *bst* phenotype that was potentiated by additional disruption of *ERP1* (Fig. 2C). Although Erv29p does not participate directly in the trafficking of GPI-APs (18), an *erv29Δ* mutation exacerbated the Gas1p processing defect observed in *erp1Δ* cells (Fig. 2D and fig. S4A).

Because most core *bst* mutants shared defects in ER export of Gas1p, we considered the possibility that broad depletion of nonessential cargoes could permit more efficient capture of essential proteins, enabling cell survival even when the COPII coat is compromised. However, maturation of the soluble vacuolar hydrolase, CPY, was normal in *bst* mutant strains (Fig. 2D and fig. S4C), suggesting that the *bst* mutations do not generally enhance secretion. Furthermore, all of the known cargo adaptors were screened in our genetic analyses, yet only the p24 proteins and Erv29p arose as hits (table S1), suggesting that

there is something unique about the cargo molecules handled by these proteins that permits deletion of Sec13p. Both GPI-APs and p24 proteins are particularly abundant (20) and highly asymmetrical membrane proteins, with the bulk of their mass residing exclusively (GPI-APs) or predominantly (p24s) in the ER lumen. Localized concentration of such asymmetrically distributed cargoes could alter the physical properties of the membrane by steric or entropic mechanisms and oppose the curvature enforced by the COPII coat by virtue of this topology (fig. S6). This model could also explain the *bst* phenotype of *erv29Δ*, because Erv29p becomes asymmetric when bound to its soluble clients. The decrease in local concentration of cargoes associated with *bst* mutants could lower the membrane-bending energy such that the COPII coat could act in the absence of Sec13p (fig. S7). Two major curvature-inducing ER membrane proteins, Rtn1p and Yop1p (21), did not affect viability of a *bst1Δ sec13Δ* strain (Fig. 3A), suggesting that global curvature of the ER membrane is unrelated to Sec13p bypass. Furthermore, deletion of the very-long-chain fatty acid elongases, Sur4p or Fen1p, reversed the viability of the *bst1Δ sec13Δ* strain but not the *bst1Δ sec13Δ-DK* strain (Fig. 3B), suggesting that these lipid species modulate curvature at the nuclear pore, where Sec13p functions in complex with Nup145 (6), rather than in COPII vesicles (22). Thus, *BST* genes appear to specifically influence membrane properties at the sites of vesicle formation.

Our genetic data point to a role for Sec13p in generating vesicles from membranes that contain the full complement of asymmetrically distributed proteins, cargoes that may confer local negative spontaneous curvature. We investigated whether Sec13p contributes rigidity to the Sec31p rods such that cage polymerization can exert sufficient

Fig. 3. Global ER curvature is unrelated to Sec13p bypass. (A) Reticulons, which maintain curvature of the ER, are not required for the *bst* phenotype because *bst1Δ sec13Δ* cells are viable when Yop1p and Rtn1p are deleted. (B) Likewise, long-chain fatty acid synthases Sur4p and Fen1p are not required for generation of curvature during COPII vesicle formation.

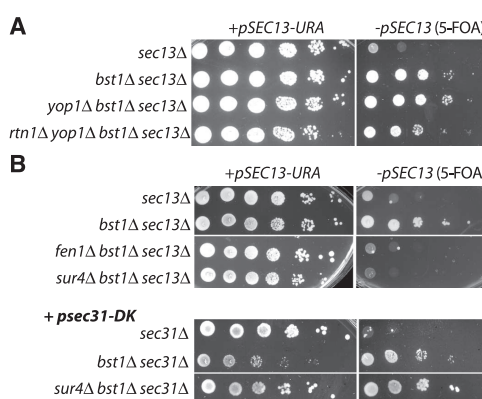
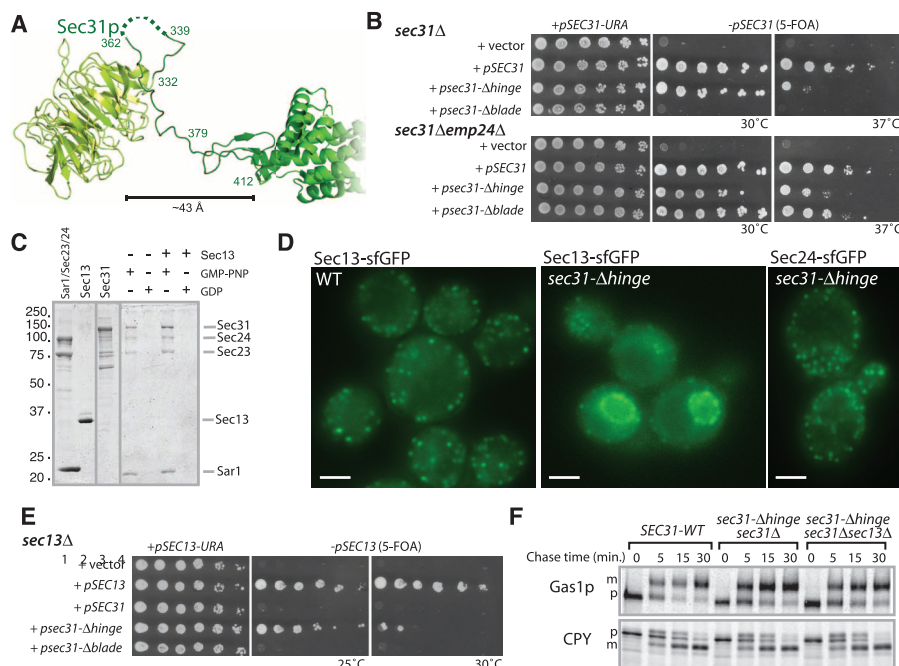


Fig. 4. A Sec31p mutant with altered flexibility suggests that Sec13p provides rigidity to the Sec13/31p complex. (A) Structural representation of Sec31p showing the flexible span between the β -propeller and ACE domains, including a region (dotted line) not visualized in the crystal structure. (B) Plasmids encoding Sec31p mutants—*psec31-Δblade* ($\Delta 379-412$) or *psec31-Δhinge* ($\Delta 332-412$)—were introduced into *sec13Δ* and *sec13Δ emp24Δ* strains as indicated and grown on 5-FOA at 30°C and 37°C. (C) Sec31p- Δ hinge was recruited to COPII-containing liposomes as described in Fig. 1D. (D) Sec13p-sfGFP (left and middle) redistributed from ER exit sites in wild-type cells (left) to the nuclear envelope and cytoplasm in a *sec13Δ-Δhinge* mutant (middle); Sec24p-sfGFP localized normally in a *sec13Δ* strain expressing Sec31p- Δ hinge (right). Scale bar, 2 μ m. (E) The *sec13Δ-Δhinge* mutant complements a *sec13Δ* strain, permitting growth on 5-FOA at 25°C. (F) Maturation of Gas1p and CPY maturation was assessed as described in Fig. 2D.



force on the ER membrane to overcome the membrane-bending energy of this asymmetric membrane. We deleted the flexible region that links the ACE and β -propeller domains of Sec31p, creating an edge element that might be expected to exhibit structural rigidity conferred by direct apposition of the two domains (Fig. 4A). Indeed, the corresponding mutant, *sec31- Δ hinge*, supported viability independent of a *bst* mutation (Fig. 4B). In contrast, deletion of the DIM alone, which preserves a short flexible domain and a disordered loop missing from the crystal structure, did not suffice for viability unless an additional *bst* mutation was present (*sec31- Δ blade* in Fig. 4B). The purified Sec31p- Δ hinge protein assembled on synthetic liposomes (Fig. 4C), suggesting robust functionality. In cells expressing only this rigidified form of Sec31p, Sec13p-GFP redistributed from large ER exit sites to a diffuse cytosolic localization with a coincident increase in nuclear envelope fluorescence (Fig. 4D). Small residual puncta may have reflected a Sec16p-associated pool of Sec13p (7), which is not required for viability because, unexpectedly, *SEC13* could be deleted entirely when Sec31p- Δ hinge was expressed (Fig. 4E). ER exit sites, as marked by Sec24p-GFP, were normal in *sec31- Δ hinge* mutant cells that lacked Sec13p (Fig. 4D), and secretory protein maturation in vivo was indistinguishable from wild-type cells (Fig. 4F), suggesting that ER export was fully operational in the absence of Sec13p. We tested the effect of converse mutations in Sec31p—those that might decrease rigidity of the Sec13/31p complex by destabilizing the β -propeller interface—and demonstrated that one such mutant was viable only in a *sec31 Δ emp24 Δ* background, despite being able to assemble into a Sec13/31p complex (fig. S8). We thus propose that Sec13p rigidifies the COPII cage, increasing its membrane-bending capacity; when a *bst* mutation creates a donor membrane more permissive to deformation, this function of Sec13p is no longer required (fig. S7).

By exploiting the *bst* phenotype to understand how secretion occurs when the COPII coat is compromised, we determined that cargo sorting can impact vesicle formation. We propose that *bst* mutations create a locally altered membrane that lacks the full complement of asymmetrically distributed cargo proteins such that the membrane can be deformed into a small vesicle absent the rigidifying effect of Sec13p. Indeed, a truncated form of Sec31p that lacks a flexible domain encompassing the Sec13p interaction motif supports viability in the absence of a *bst* mutation, suggesting that an artificially rigidified coat can exert sufficient force to bend an asymmetric cargo-rich membrane. Membrane-bending properties of coat proteins have largely been characterized on synthetic liposomes (23–25), which fail to recapitulate important properties of cellular membranes, including the abundance and complexity of protein cargoes and the asymmetry that lipids and proteins can exhibit across the bilayer. Coat proteins have evolved to overcome

the barrier to curvature that such constituents present, employing multiple mechanisms to enforce shape changes (4). The structural rigidity conferred to the COPII coat by Sec13 has parallels in other vesicle transport systems, notably the clathrin coat, where structural models predict that depletion of clathrin light chain renders the heavy-chain triskelion more flexible, yet still competent for vesicle formation (26). The presence of Sec13 within the COPII cage may also permit multiple geometries, allowing the coat to adapt to cell- or condition-specific cargo packaging requirements (27). Indeed, mammalian cells depleted of Sec13 show collagen-specific trafficking defects (28) that may reflect the inability of a Sec13-free coat to adequately deform the membrane around a uniquely rigid cargo. Furthermore, ER export of large cargoes requires alternative COPII subunits like the yeast Sec24p paralog Lst1p/Sfb2p, which facilitates traffic of the abundant oligomeric Pma1p complex (29, 30), or accessory factors such as human TANGO1, which promotes trafficking of procollagen (31). Such accessory proteins could influence membrane properties to either oppose the force of the COPII coat, preventing premature vesicle scission, or augment curvature conferred by the coat to ensure encapsulation of a rigid cargo. In addition to large and rigid cargoes, we propose that proteins with particularly asymmetric topologies will also influence the mechanics of vesicle formation.

References and Notes

1. E. A. Miller, C. Barlowe, *Curr. Opin. Cell Biol.* **22**, 447 (2010).
2. X. Bi, R. A. Corpina, J. Goldberg, *Nature* **419**, 271 (2002).
3. S. M. Stagg *et al.*, *Nature* **439**, 234 (2006).
4. J. Zimmerberg, M. M. Kozlov, *Nat. Rev. Mol. Cell Biol.* **7**, 9 (2006).
5. S. Fath, J. D. Mancias, X. Bi, J. Goldberg, *Cell* **129**, 1325 (2007).
6. S. G. Brohawn, T. U. Schwartz, *Nat. Struct. Mol. Biol.* **16**, 1173 (2009).
7. J. R. R. Whittle, T. U. Schwartz, *J. Cell Biol.* **190**, 347 (2010).
8. M. J. Elrod-Erickson, C. A. Kaiser, *Mol. Biol. Cell* **7**, 1043 (1996).

9. M. Marzioch *et al.*, *Mol. Biol. Cell* **10**, 1923 (1999).
10. Materials and methods are available as supporting material on Science Online.
11. C. Barlowe *et al.*, *Cell* **77**, 895 (1994).
12. A. H. Tong *et al.*, *Science* **294**, 2364 (2001).
13. M. Fujita, Y. Jigami, *Biochim. Biophys. Acta* **1780**, 410 (2008).
14. M. Fujita *et al.*, *Cell* **139**, 352 (2009).
15. M. Muñiz, C. Nuoffer, H. P. Hauri, H. Riezman, *J. Cell Biol.* **148**, 925 (2000).
16. M. Fujita *et al.*, *J. Cell Biol.* **194**, 61 (2011).
17. A. Copic *et al.*, *Genetics* **182**, 757 (2009).
18. W. J. Belden, C. Barlowe, *Science* **294**, 1528 (2001).
19. J. Dancourt, C. Barlowe, *Annu. Rev. Biochem.* **79**, 777 (2010).
20. S. Ghaemmaghami *et al.*, *Nature* **425**, 737 (2003).
21. G. K. Voeltz, W. A. Prinz, Y. Shibata, J. M. Rist, T. A. Rapoport, *Cell* **124**, 573 (2006).
22. R. Schneider *et al.*, *Biochem. J.* **381**, 941 (2004).
23. M. C. S. Lee *et al.*, *Cell* **122**, 605 (2005).
24. A. Bielli *et al.*, *J. Cell Biol.* **171**, 919 (2005).
25. B. J. Peter *et al.*, *Science* **303**, 495 (2004).
26. J. D. Wilbur *et al.*, *Dev. Cell* **18**, 854 (2010).
27. S. M. Stagg *et al.*, *Cell* **134**, 474 (2008).
28. A. K. Townley *et al.*, *J. Cell Sci.* **121**, 3025 (2008).
29. K. J. Roberg, M. Crotwell, P. Espenshade, R. Gimeno, C. A. Kaiser, *J. Cell Biol.* **145**, 659 (1999).
30. Y. Shimoni *et al.*, *J. Cell Biol.* **151**, 973 (2000).
31. K. Saito *et al.*, *Cell* **136**, 891 (2009).

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Materials and Methods
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Influence of Synaptic Vesicle Position on Release Probability and Exocytotic Fusion Mode

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Neurotransmission depends on movements of transmitter-laden synaptic vesicles, but accurate, nanometer-scale monitoring of vesicle dynamics in presynaptic terminals has remained elusive. Here, we report three-dimensional, real-time tracking of quantum dot-loaded single synaptic vesicles with an accuracy of 20 to 30 nanometers, less than a vesicle diameter. Determination of the time, position, and mode of fusion, aided by trypan blue quenching of Qdot fluorescence, revealed that vesicles starting close to their ultimate fusion sites tended to fuse earlier than those positioned farther away. The mode of fusion depended on the prior motion of vesicles, with long-dwelling vesicles preferring kiss-and-run rather than full-collapse fusion. Kiss-and-run fusion events were concentrated near the center of the synapse, whereas full-collapse fusion events were broadly spread.

Neurons communicate by releasing neurotransmitter and thus activating postsynaptic receptors. The position and movement

of neurotransmitter-containing vesicles are essential for proper communication. However, vesicle dynamics during the period leading up to release

remain mysterious. Uncertainty exists about the possible role of vesicle position in the determination of vesicle fusion probability (1). A controversy concerns the possible existence of two modes of fusion—full-collapse fusion (FCF) or kiss-and-run (K&R)—reported in central nervous system (CNS) neurons (2–6), but having an uncertain relation between vesicle movement and fusion location—especially in three dimensions (7). Recently, several methods have been devel-

oped to localize fluorescent molecules in three dimensions (8–10) but real-time three-dimensional (3D) localization has not been applied to synaptic vesicles in living neurons.

To localize single synaptic vesicles in real-time and in three dimensions, we built a microscope with dual-focus imaging optics, modified from a published design (9) (fig. S1). The accuracy of the localization (2.35σ , corresponding to the full-width at half maximum) was ~ 20 nm for x - and y - and ~ 30 nm for z -localization (Fig. 1A) (for 10 Hz imaging, standard in this paper). Real-time capabilities were tested by imposing stepwise movements on single Qdot-containing vesicles in a fixed neuron. For z axis displacements of 40 nm (~ 1 vesicle diameter), the estimated z axis displacement for individual frames was 42 ± 2.7 nm (SEM)

($n = 10$) (Fig. 1B), an accuracy well below a single vesicle diameter.

Single vesicles were efficiently labeled by use of streptavidin-coated Qdots conjugated to biotinylated antibodies against the luminal domain of the vesicular protein synaptotagmin 1. In an exemplar 3D trajectory (Fig. 1C), a single vesicle in a living neuron underwent ~ 12 s of intense movement, travelling almost unidirectionally with the net displacement of $3.2\ \mu\text{m}$ over 90 s of imaging, with dwelling in two discrete zones, presumptive presynaptic terminals marked by distinct clouds of vesicles labeled with FM 4-64, a lipophilic probe for vesicular turnover.

Exocytosis of the Qdot-loaded vesicles was registered by a sudden drop in the fluorescence, caused by uptake of an extracellular quencher,

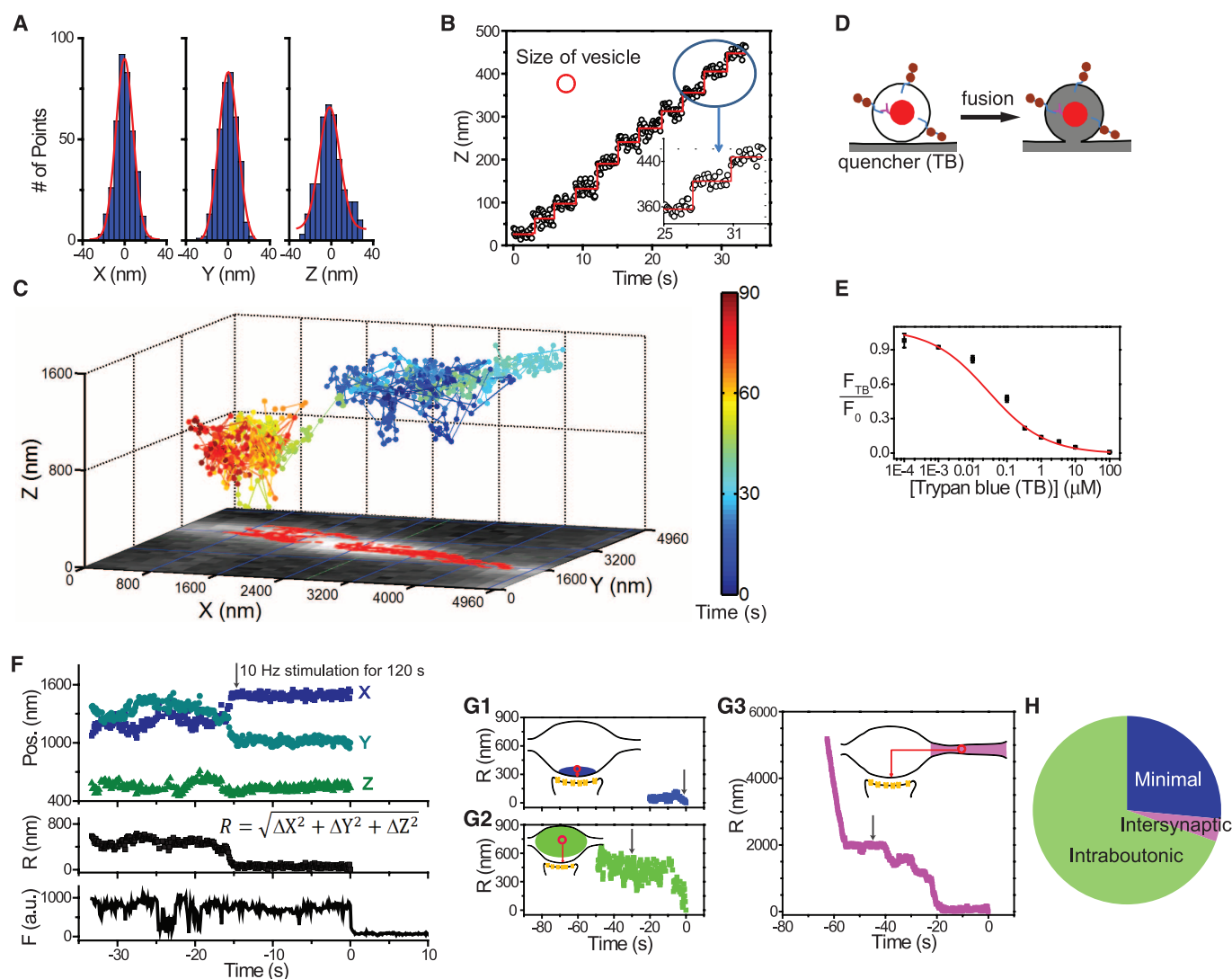


Fig. 1. Real-time, 3D tracking of single vesicles. (A) Immobilized Qdot (10-Hz imaging). Positional estimates had standard deviations (σ) of 8.5 nm (x), 8.5 nm (y), and 12.3 nm (z). (B) Tracking z -axis, 40-nm movements of a single Qdot in a fixed neuron. Red circle, approximate size of a vesicle. (Inset) Expanded view. (C) 3D trace of Qdot-labeled vesicle in living neuron, with x - y plane projection (red squares), overlaid on image of FM 4-64-labeled vesicles (white). Color bar, elapsed time. Stimulation (10 Hz, 120 s) started at 20 s; vesicle exocytosed at 90 s.

(D) Quenching of Qdot fluorescence by TB pinpoints the moment of exocytosis. (E) Dependence of unquenched fraction (F_{TB}/F_0) on TB concentration. (F) 3D position and fluorescence of Qdot-labeled vesicle (exocytosis, $t = 0$). (G) Traces and representations of minimal, intraboutonic, and intersynaptic patterns of vesicle movement before exocytosis (G1 to G3, respectively). Average latencies to fusion were 40.7, 42.9, and 55.5 s, respectively. Gray arrows, initiation of 1200 APs stimulation. (H) Prevalence of three patterns of movement.

trypan blue (TB) (6, 11) (Fig. 1D). We used 1 μ M TB in most experiments, guided by the concentration dependence of TB quenching of Qdots (F_{TB}/F_0) (Fig. 1E). Neither the quencher nor loading with antibody-conjugated Qdots affected vesicle dynamics, as assessed by uptake and destaining of FM 4-64 (fig. S2).

We analyzed 3D positions over time, calculating the momentary radial distance to the eventual location of fusion ($R = \sqrt{\Delta X^2 + \Delta Y^2 + \Delta Z^2}$). In one example (Fig. 1F), a tracked vesicle was mo-

bile for the first 18 s of imaging but then remained stationary at the fusion site ($R \approx 0$) for another 15 s before undergoing fusion ($t = 0$). The fluorescence trace included a blinking event (-24 s), which confirmed that the signal arose from a single Qdot, and displayed a sharp drop 14 s after the onset of field stimulation, which corresponded to complete equilibration of the vesicle lumen with the quencher-containing external solution. Similar traces were obtained from γ -aminobutyric acid (GABA)-releasing (GABAergic) terminals, by

using Qdots conjugated to biotinylated antibodies against the luminal domain of vesicular GABA transporter (VGAT) (fig. S3A).

Single vesicles displayed three patterns of movement before exocytosis—intersynaptic, intraboutonic, and minimal (residing very close to the fusion site for the entire observation period). Intersynaptic movement was categorized by a net displacement of >1 μ m (Fig. 1, C and G3), consistent with vesicle sharing between nearby synapses (12), and was the least prevalent [3 out of 81 (4%)] (Fig. 1H). Intraboutonic movement, defined by net displacements between 0.1 μ m and 1 μ m (Fig. 1G2), was the most abundant pattern [56 out of 81 (69%)], presumably because it typifies the bulk of recycling vesicles. Net movements of <0.1 μ m before exocytosis (Fig. 1G1), categorized as minimal, were observed in 22 of 81 (27%) of cases.

Next, we tested whether proximity to release sites is a critical determinant of vesicle-release probability (Fig. 2). This long-held assumption (1, 13) predicts that, on average, a vesicle starting off close to its ultimate release site will fuse earlier than one located further away. Accordingly, we directly determined the net 3D displacement from the starting position of vesicles to their fusion sites, information unobtainable from static electron microscopy (EM) images. The net displacement to fusion site proved to be a strong predictor of the latency to fusion ($P < 0.005$) (fig. S4). Vesicles with relatively high release probability (high P_{TV}) were defined by a brief latency to release (<20 s after onset of stimulation), whereas vesicles with relatively low P_{TV} were discerned by long latency (>50 s after onset of

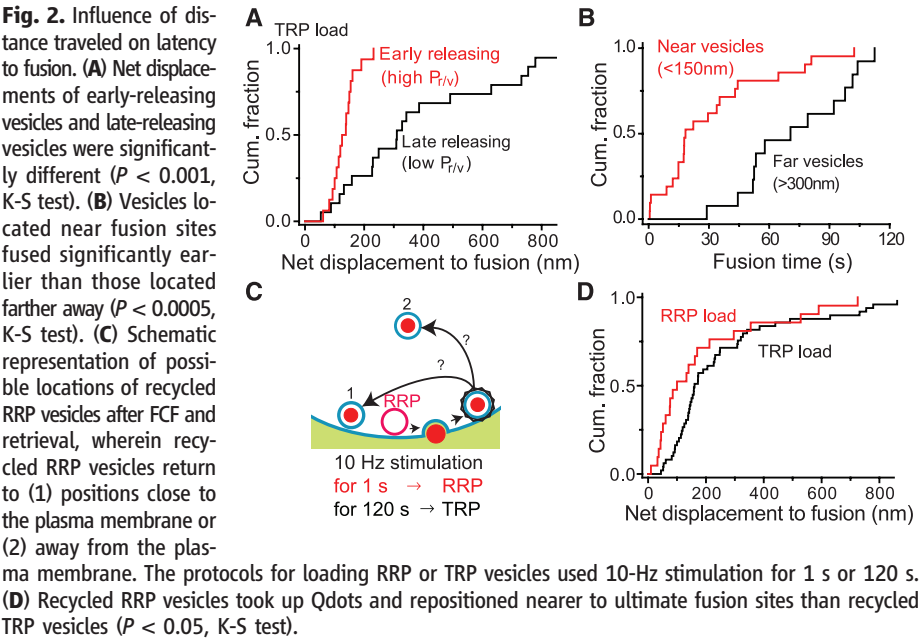
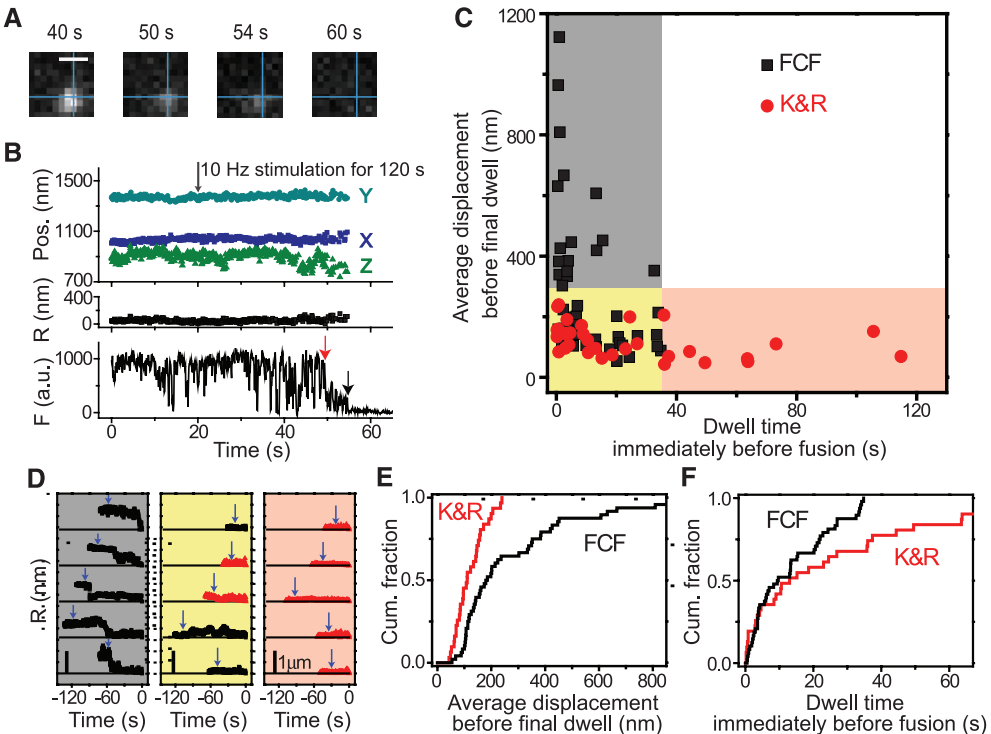


Fig. 3. Detection of K&R by partial quenching and relation of fusion mode to prior motion. (A) Images taken before fusion (40 s), after partial quenching (50 s), and before (54 s) and after full quenching (60 s). The intersection of the perpendicular lines marked the position before the first fusion. Bar, 0.8 μ m. (B) 3D position and fluorescence of Qdot-loaded vesicle in (A). Red arrow, K&R; black arrow, final FCF event. (C) Relation of fusion mode to final dwell time and average displacement preceding final dwell. (D) Traces exemplifying three categorized groups. Blue arrows, stimulation initiation. Bar, 1 μ m. (E) Average displacements before final dwell were significantly smaller with K&R (average, 105 ± 10.8 nm, $n = 22$) than with FCF (average, 278 ± 34.2 nm, $n = 48$) ($P < 0.005$, K-S test). (F) Dwell times at the final position were significantly longer with K&R (average, 30.3 ± 7.01 s) than with FCF (average, 10.7 ± 1.55 s) ($P < 0.001$, K-S test).



stimulation). The high P_{rv} vesicles traveled a shorter distance to fusion sites (median, 125 nm, $n = 15$) than low P_{rv} vesicles (median, 310 nm, $n = 19$) [$P < 0.001$, Kolmogorov–Smirnov (K-S) test] (Fig. 2A). Likewise, vesicles starting near fusion sites (<150 nm) underwent exocytosis earlier (median, 18 s; average, 31 ± 6.2 s; $n = 21$) than those starting further away (>300 nm) (median, 71 s; average, 73 ± 7.4 s; $n = 13$) ($P < 0.0005$, K-S test) (Fig. 2B). Thus, proximity is a key factor in determining vesicle release probability even for vesicles not already docked to their release site.

To determine whether a vesicle's pool of origin influences its eventual position after FCF and retrieval or if vesicles become randomly dispersed (Fig. 2C), we investigated the positions of the readily releasable pool (RRP) and total recycling pool (TRP) of vesicles after recycling, in experiments akin to those in previous EM studies (14, 15). Labeling of vesicles was either sharply restricted to vesicles from the RRP {10 Hz for 1 s [10 action potential (AP) stimulation]} or spread across the TRP (1200 APs). Vesicles originating from the RRP relocated at positions significantly closer to the fusion sites (median 100 nm, $n = 21$) (Fig. 2D) than vesicles derived from the TRP (median 160 nm, $n = 49$)

(K-S test, $P < 0.05$) [see also (15)]. The observed difference in spatial distribution was remarkable, because Qdots (unlike FM dye) can only be taken up after FCF and clathrin-mediated retrieval (Fig. 2C) (5). Nevertheless, vesicles with a high probability of release in a first round of exocytosis behaved detectably differently compared with recycling vesicles as a whole, consistent with a role for molecular determinants as contributors to pool identity (14). Such determinants may apply to RRP vesicles that reside far enough from the fusion site to be strictly undocked (1, 13, 14).

The degree of quenching of Qdot fluorescence by TB distinguished FCF and K&R. TB should completely equilibrate after FCF but only partially equilibrate during a brief fusion pore opening of K&R. Partial quenching of a single Qdot due to K&R is illustrated in Fig. 3A (see detailed analyses in SOM and fig. S5). The fluorescence trace showed a sudden, irreversible, but only partial, drop (red arrow), falling from an initial level (defined as 1.0) to a partially quenched level ($F_{TB}/F_0 = 0.312$), significantly larger than expected for Qdots steadily exposed to 1 μ M TB [0.137 ± 0.003 (SEM), $n = 3$]. After a sojourn of ~ 5 s, the fluorescence dropped further (black arrow), to a level of virtually zero, presumably in association with Qdot movement out of the region

of interest after full fusion. Similar two-step losses of fluorescence, apparently K&R and FCF events in succession, were observed with single vesicles in inhibitory nerve terminals, tracked with VGAT-antibody-conjugated Qdots (fig. S3B). We verified that partial quenching denoted K&R fusion by using a lower quencher concentration and observing a milder degree of partial quenching (fig. S6A).

On many occasions, the Qdot-labeled vesicle remained stationary before undergoing K&R (Fig. 3B), which raised the question of whether the fusion mode is influenced by prior motion. To investigate this, we determined the final dwell time before fusion and the average displacement before that final dwell. These parameters varied between the two fusion modes (Fig. 3C; specific examples in Fig. 3D). Vesicles located close (<300 nm) to their fusion sites with dwell times longer than 35 s (pink shading) invariably underwent K&R. In contrast, vesicles that approached from a long distance (>300 nm) before the final dwell time (gray shading) consistently underwent FCF. Vesicles characterized by both a brief dwell time and previously close proximity to fusion sites showed mixed results (yellow shading). Analysis of pooled data (Fig. 3, E and F) confirmed that vesicles undergoing K&R traveled shorter distances to reach fusion sites than those undergoing FCF ($P < 0.005$, K-S test), whereas vesicles undergoing K&R remained stationary at fusion sites for longer than those undergoing FCF ($P < 0.001$, K-S test). Thus, determination of fusion mode is not a strictly stochastic decision at the time of exocytosis but is strongly dependent on the prior history of vesicle position.

Next, we pinpointed the locus of fusion relative to pre- and postsynaptic markers (Fig. 4, A and B): FM 4-64-stained presynaptic vesicles (red) and postsynaptic density protein PSD-95 fused to green fluorescent protein (PSD-95-GFP) (green). The “synaptic axis,” defined by a vector connecting centroids of pre- and postsynaptic markers ($\rightarrow$, Fig. 4, A and C), was presumed to pass roughly through the center of the active zone. The angle (θ , Fig. 4A) between the synaptic axis and the z axis of the microscope was 122° in Fig. 4B (average, $126 \pm 3.3^\circ$, $n = 31$). The pre- and postsynaptic landmarks provided a spatial context for the pre-exocytosis motion of Qdot-labeled vesicles (Fig. 4C). The trajectory of an exemplar vesicle began near the center of FM 4-64 staining but ended at a site of exocytosis near the centroid labeled with PSD-95-GFP. Just before fusion by FCF (55.7 s) (black circle, Fig. 4C), the vesicle was 314 nm away from the synaptic axis ($r = 314$ nm). Collectively, FCF events appeared evenly distributed over a disc centered on the synaptic axis, the cumulative distribution of events increasing with the square of distance (r^2) (dashed line, Fig. 4D) [coefficient of determination (R^2) = 0.94; $r_{\text{median}} = 295$ nm; $n = 22$]. In contrast, K&R events occurred closer to the synaptic axis ($P < 0.001$, K-S test), with $r_{\text{median}} = 109$ nm, as if kept within a smaller zone ($R^2 = 0.87$ for parabolic fit, $n = 9$). The con-

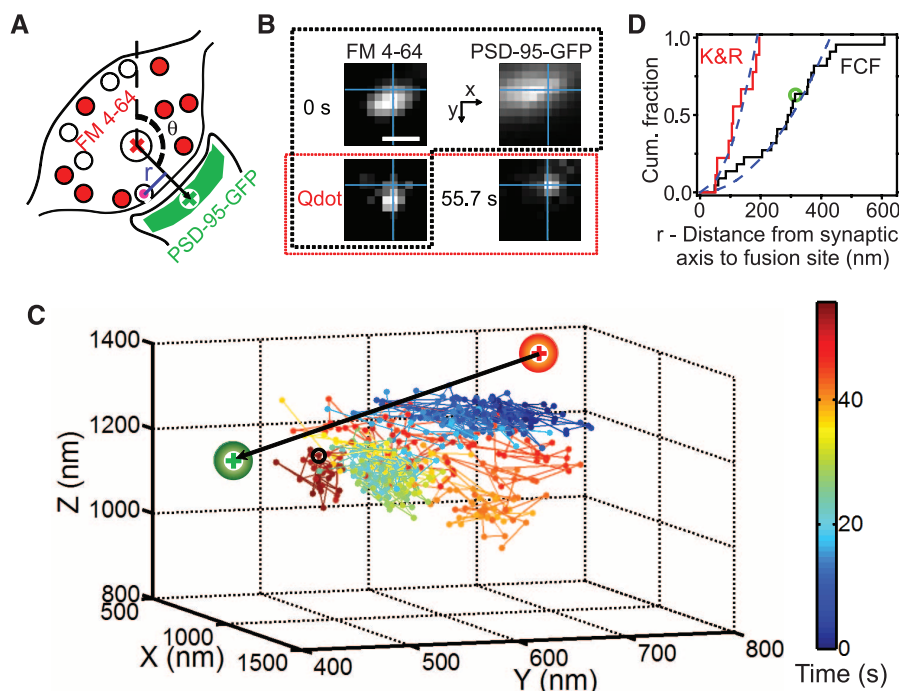


Fig. 4. Relation between fusion mode and proximity to synaptic axis (r). (A) Synaptic axis ($\rightarrow$) defined as a vector connecting centroids of FM 4-64 (+) and PSD-95-GFP (+), pre- and postsynaptic markers. r defined as orthogonal distance from fusion site to synaptic axis. (B) Images of FM 4-64, PSD-95-GFP, and Qdot in the same plane, taken initially (0 s, surrounded by dashed black line) and just before fusion (55.7 s). Images of Qdot are marked by a red-outlined rectangle. The intersection of the perpendicular lines marked the last position before fusion. Bar, 0.8 μ m. (C) 3D trajectory of the vesicle in (B) relative to FM 4-64 and PSD-95-GFP centroids. Color bar, elapsed time; 10-Hz stimulation starting at 20 s. Black circle, the last position before fusion. (D) Differing cumulative distributions of r for two fusion modes. Dashed line shows parabolic fit, corresponding to uniform distribution. Circle indicates the vesicle in Fig. 4, B and C.

finement of K&R events near the center of the presynaptic terminal would put glutamate release by K&R in alignment with *N*-methyl-D-aspartate receptors (NMDARs) located close to the center of the postsynaptic density but in less direct apposition with most of the fast α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA), which appear dominant over NMDARs at the periphery (fig. S7) [(16, 17) but see also (18)]. Differences in alignment are important because (i) lateral spread of neurotransmitter causes rapid dilution of peak [glutamate] (19), with a lateral space constant of ~ 125 nm (20) and (ii) AMPARs require millimolar [glutamate] for activation (21). Thus, our findings predict that exocytosis by K&R should strongly activate NMDARs but cause uneven activation of AMPARs, whereas both receptor types should be robustly activated by FCF, as recently found [(4), see also (22)].

Our real-time 3D tracking of single synaptic vesicles in living hippocampal nerve terminals enabled direct observation of the motion of single vesicles toward their fusion sites. It also revealed unexpected relations between the trajectory of vesicles, the location of fusion, and their mode of exocytosis.

References and Notes

1. S. O. Rizzoli, W. J. Betz, *Nat. Rev. Neurosci.* **6**, 57 (2005).
2. A. M. Aravanis, J. L. Pyle, R. W. Tsien, *Nature* **423**, 643 (2003).
3. S. P. Gandhi, C. F. Stevens, *Nature* **423**, 607 (2003).
4. D. A. Richards, *J. Physiol.* **587**, 5073 (2009).
5. Q. Zhang, Y. Li, R. W. Tsien, *Science* **323**, 1448 (2009).
6. N. C. Harata, S. Choi, J. L. Pyle, A. M. Aravanis, R. W. Tsien, *Neuron* **49**, 243 (2006).
7. P. Vanden Berghe, J. Klingauf, *J. Physiol.* **572**, 707 (2006).
8. B. Huang, W. Wang, M. Bates, X. Zhuang, *Science* **319**, 810 (2008).
9. T. M. Watanabe, T. Sato, K. Gonda, H. Higuchi, *Biochem. Biophys. Res. Commun.* **359**, 1 (2007).
10. G. Shtengel *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 3125 (2009).
11. M. Howarth *et al.*, *Nat. Methods* **5**, 397 (2008).
12. K. J. Darcy, K. Staras, L. M. Collinson, Y. Goda, *Nat. Neurosci.* **9**, 315 (2006).
13. A. Denker, S. O. Rizzoli, *Front. Synaptic Neurosci.* **2**, 135 (2010).
14. S. O. Rizzoli, W. J. Betz, *Science* **303**, 2037 (2004).
15. T. Schikorski, C. F. Stevens, *Nat. Neurosci.* **4**, 391 (2001).
16. V. N. Kharazia, R. J. Weinberg, *Neurosci. Lett.* **238**, 41 (1997).
17. P. Somogyi, Tamás, R. Lujan, E. H. Buhl, *Brain Res. Res. Rev.* **26**, 113 (1998).
18. A. Dani, B. Huang, J. Bergan, C. Dulac, X. Zhuang, *Neuron* **68**, 843 (2010).
19. V. V. Uteshev, P. S. Pennefather, *Biophys. J.* **72**, 1127 (1997).
20. K. M. Franks, C. F. Stevens, T. J. Sejnowski, *J. Neurosci.* **23**, 3186 (2003).
21. J. S. Diamond, C. E. Jahr, *J. Neurosci.* **17**, 4672 (1997).
22. S. Choi, J. Klingauf, R. W. Tsien, *Philos. Trans. R. Soc. London B Biol. Sci.* **358**, 695 (2003).

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Decoding in the Absence of a Codon by tmRNA and SmpB in the Ribosome

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In bacteria, ribosomes stalled at the end of truncated messages are rescued by transfer-messenger RNA (tmRNA), a bifunctional molecule that acts as both a transfer RNA (tRNA) and a messenger RNA (mRNA), and SmpB, a small protein that works in concert with tmRNA. Here, we present the crystal structure of a tmRNA fragment, SmpB and elongation factor Tu bound to the ribosome at 3.2 angstroms resolution. The structure shows how SmpB plays the role of both the anticodon loop of tRNA and portions of mRNA to facilitate decoding in the absence of an mRNA codon in the A site of the ribosome and explains why the tmRNA-SmpB system does not interfere with normal translation.

Transfer-messenger RNA (tmRNA), also known as 10S RNA or SsrA, is a highly structured RNA that combines properties of transfer RNA (tRNA) and mRNA in one molecule about 350 nucleotides long (Fig. 1A) (1, 2). The tRNA-like domain (TLD) of tmRNA lacks an

anticodon stem loop but contains an acceptor arm (3) that can be aminoacylated at its 3' end by the same alanyl tRNA synthetase that aminoacylates tRNA^{Ala}. A different region of tmRNA contains a short internal open reading frame (ORF) that acts as an mRNA template. In addition, tmRNA contains several pseudoknots and helices.

Ribosomes that reach the end of prematurely truncated or defective messages are stalled because the absence of a complete codon in the A site prevents either elongation or normal termination. In bacteria, they are rescued by tmRNA in a process called *trans*-translation because it involves continuing translation by changing the mRNA template. In this process, elongation factor Tu (EF-Tu) delivers tmRNA to the A site of

the stalled ribosome. The nascent polypeptide chain is transferred to the alanine on the TLD. Subsequently, translocation brings the first codon of the ORF into the A site of the ribosome, and translation resumes with the ORF as the mRNA (2). The short sequence coded by the ORF thus added to the C terminus of the partially synthesized protein acts as a degradation tag (4). Therefore, tmRNA acts both to rescue ribosomes and to target incompletely synthesized proteins for degradation.

The binding of tmRNA to stalled ribosomes requires the protein SmpB (5), which can bind to tmRNA simultaneously with EF-Tu (6). Crystal structures of SmpB in complex with the TLD suggest that the protein substitutes for the missing anticodon stem inside the ribosome (7, 8), which was supported by electron microscopy (EM) studies at ~ 15 Å resolution (9, 10). A previous EM study found two molecules of SmpB with tmRNA in the ribosome, with the C terminus of one of them near the decoding center of the 30S ribosomal subunit (11). The observed proximity to the decoding center agrees with hydroxyl radical and chemical probing experiments that show protection of 16S ribosomal RNA (rRNA) bases A1492, A1493, and G530 at the decoding center upon binding of the tmRNA-SmpB complex to the ribosome (*Escherichia coli* numbering used for rRNA throughout) (12, 13). However, mutations at these positions do not reduce SmpB binding to the decoding site (13) or reduce the rate of peptidyl transfer onto tmRNA (14). The mechanism by which tmRNA and SmpB acting in concert can facilitate “decoding” in the absence of codon-anticodon base pairing has remained unclear.

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Here, we present the crystal structure of the *Thermus thermophilus* ribosome bound to a complex consisting of a fragment of tmRNA (tmRNA^{Δm}) along with SmpB and EF-Tu trapped in the guanosine diphosphate (GDP) state im-

mediately after guanosine triphosphate (GTP) hydrolysis by using the antibiotic kirromycin, as was done previously with the tRNA complex (15). In addition, the ribosome contains deacylated tRNA^{fMet} in the P and E sites of

the ribosome and a truncated mRNA that contains two bases in the A site after the P-site codon. The construct tmRNA^{Δm} was based on the sequence of the *ssrA* gene from *T. thermophilus* and contained 89 nucleotides (Fig. 1A). It includes the TLD but does not contain the pseudoknots or the ORF of tmRNA. Together with SmpB and EF-Tu, similar constructs of tmRNA were capable of interacting with stalled ribosomes (10) and even of synthesizing polyalanine on 70S ribosomes in the absence of an mRNA template (16).

Crystals were obtained in a new form with only one ribosome in the asymmetric unit. We carried out molecular replacement and initial refinement using an empty ribosome as a starting model [for details see supplementary online materials (SOM)]. All the components of the Ala-tmRNA^{Δm}-SmpB-EF-Tu-GDP complex were clearly visible in an initial unbiased difference Fourier map (fig. S1). Ligands were subsequently built, and the complete model was refined to 3.1 Å resolution [$I/\sigma(I) = 2.12$ at 3.20 Å] (Fig. 1B and table S1), which resulted in a final $R_{\text{work}}/R_{\text{free}}$ of 23.0%/27.0%.

The complex of Ala-tmRNA^{Δm} with SmpB and EF-Tu-GDP-kirromycin in the A site of the ribosome is shown in Fig. 1C. The model of EF-Tu is complete except for the switch I region (residues 41 to 66), which was also disordered in a previous crystal structure of EF-Tu in complex with aminoacyl-tRNA (15). The TLD of tmRNA^{Δm} was well ordered for residues 1 to 24 and 314 to 349. SmpB could be modeled completely, including the entire C terminus, which is unstructured in solution but critical for tmRNA function (17–19). The overall conformation of the ribosome closely resembles that of the equivalent complex of EF-Tu with acylated tRNA (15). Despite the absence of an anticodon in tmRNA, the 30S subunit is in a “closed” conformation that is normally characteristic of the presence of a cognate codon-anticodon base pairing in the decoding center of the 30S subunit (20, 21). The analysis of this structure now allows detailed insights into how tmRNA and SmpB together recognize stalled ribosomes and facilitate decoding, even in the absence of a codon-anticodon interaction.

The complex of SmpB and the TLD resembles a tRNA molecule. Overall, the conformation of the ribosome-bound tmRNA^{Δm}-SmpB complex shows the canonical L shape of a tRNA (Fig. 2) and is similar to a closely related isolated crystal structure (root mean square deviation of ~1.0 Å) (8). This implies that the complex of SmpB and the TLD undergoes only slight conformational changes during its binding to stalled ribosomes. The only exception is the D loop, whose 5' region moves toward the acceptor stem in the ribosome complex (fig. S2). This part of the D loop shows weaker electron density and was disordered in one of the isolated crystal structures, which indicates that it has structural flexibility (7). Overall the TLD structurally

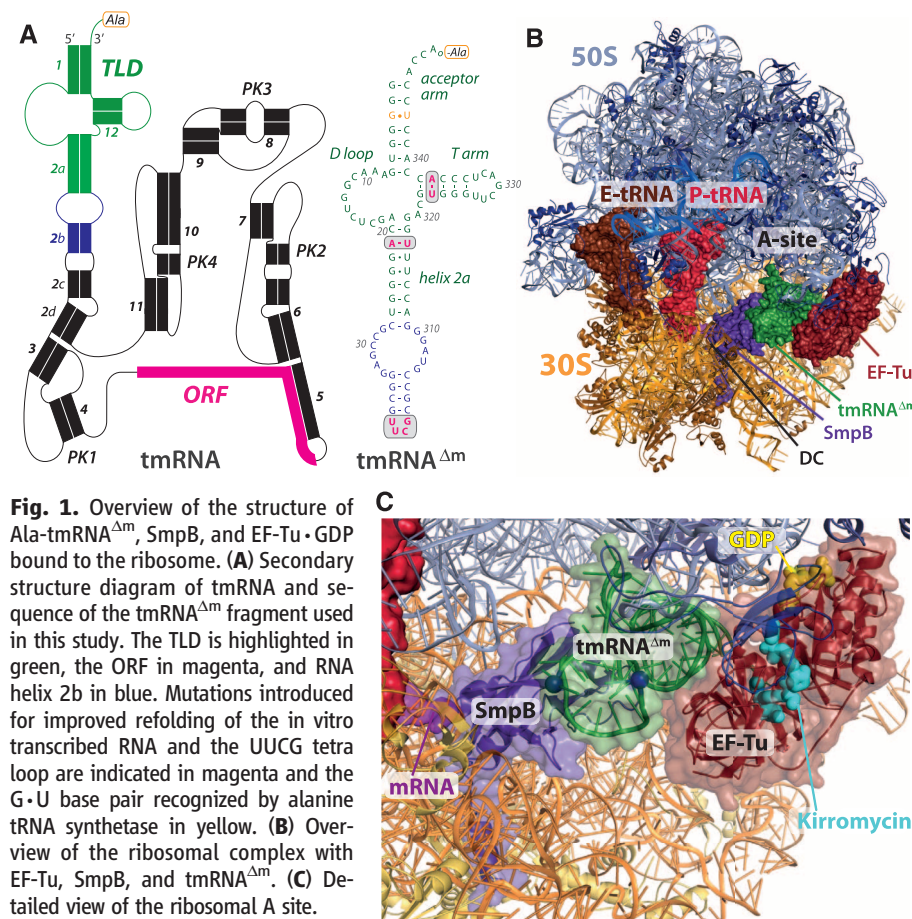
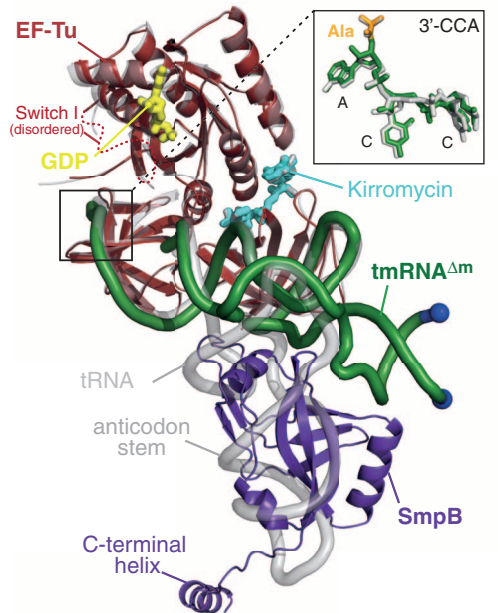


Fig. 1. Overview of the structure of Ala-tmRNA^{Δm}, SmpB, and EF-Tu-GDP bound to the ribosome. (A) Secondary structure diagram of tmRNA and sequence of the tmRNA^{Δm} fragment used in this study. The TLD is highlighted in green, the ORF in magenta, and RNA helix 2b in blue. Mutations introduced for improved refolding of the in vitro transcribed RNA and the UUCG tetra loop are indicated in magenta and the G·U base pair recognized by alanine tRNA synthetase in yellow. (B) Overview of the ribosomal complex with EF-Tu, SmpB, and tmRNA^{Δm}. (C) Detailed view of the ribosomal A site.

Fig. 2. Superposition of tmRNA^{Δm}-SmpB-EF-Tu with a complex of A-site tRNA and EF-Tu [gray, Protein Data Bank (PDB) code 2Y10] (36). In the tmRNA-SmpB complex, SmpB substitutes for the anticodon arm of tRNA. The interactions of EF-Tu (red) and the acceptor-stem region of tmRNA (green) are nearly identical to that observed for the tRNA-EF-Tu complex (gray). (Inset) A close-up view of the 3' end CCA sequence. The ends of the tmRNA^{Δm} model that connect to helix 2b point out of the ribosome and are shown as blue spheres.



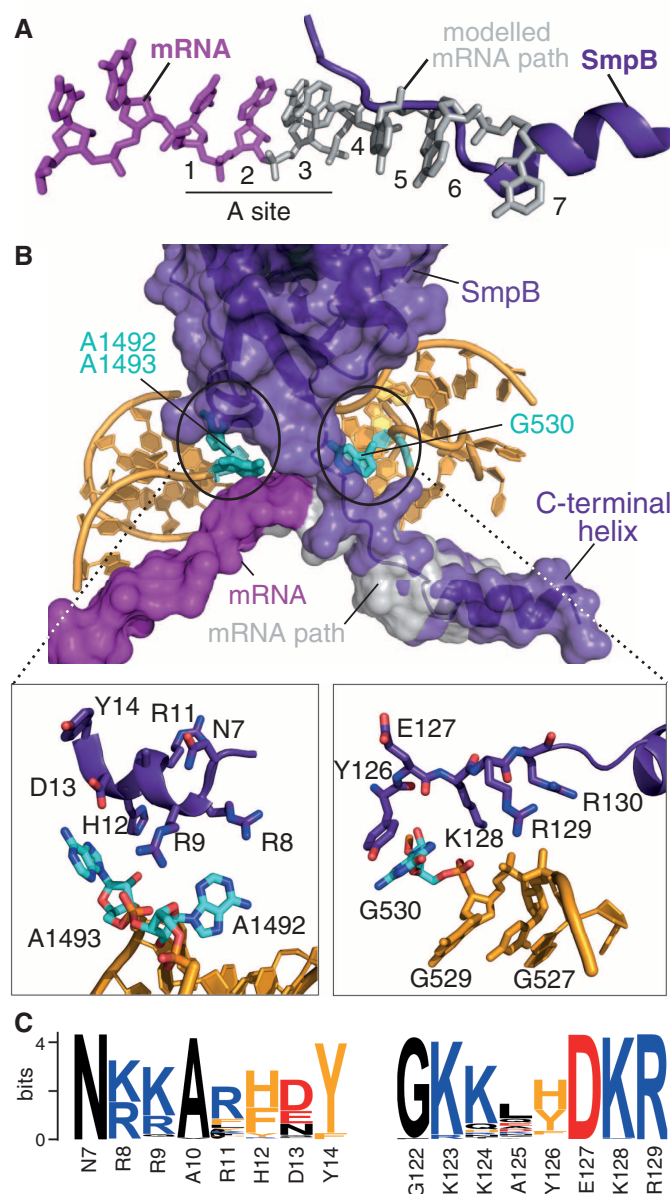
and functionally mimics the acceptor arm and T Ψ C-arm of tRNA. As suggested by previous isolated structures of the TLD-SmpB complex, the core of SmpB structurally mimics the anticodon stem of tRNA in the ribosome. Helix 2a points out of the ribosome, where its positioning would allow the pseudoknot modules of tmRNA to form an arc around the beak of the 30S subunit, as previously reported by several EM studies (22–24). The conformation of the 3'-CCA end of Ala-tmRNA^{Am} and the acceptor arm, as well as the T-arm portion that interacts with EF-Tu, closely resembles the pre-accommodation state of aminoacyl-tRNA (Fig. 2). The structure of EF-Tu in the tmRNA complex showed clear extra density for GDP and kirromycin and is nearly identical to the structure of EF-Tu with cognate aminoacyl-tRNA in the ribosome. As expected, this confirms that the addition of kirromycin has trapped the Ala-tmRNA^{Am}-SmpB particle in the state just after GTP hydrolysis in EF-Tu. Notably, SmpB shows no interaction with EF-Tu. The closest distance is about 5 Å between Y68 of SmpB and EF-Tu residue I375 (*T. thermophilus* numbering). Y68 is part of the central loop of SmpB that has high flexibility in solution and likely increases the aminoacylation efficiency of tmRNA (8). Given the EF-Tu and SmpB binding sites on the TLD, the structure does not support the simultaneous binding of two SmpB proteins to the TLD before translocation (11) but rather a model in which tmRNA and SmpB enter the ribosome as a 1:1 complex that structurally mimics a tRNA whose anticodon stem loop has been replaced by SmpB.

The C terminus of SmpB, which is unstructured in solution, is completely ordered on binding the ribosome (residues 122 to 144) (Fig. 1C). Its interactions with the ribosome are consistent with a recent hydroxyl radical probing study using SmpB mutations of the carboxy-terminal tail (25). The C terminus first extends from the SmpB core toward the ribosomal decoding base G530 and then continues along the path normally occupied by mRNA downstream of the A-site codon (Fig. 3, A and B). This part of the tail from residue G122 to D132 is well ordered and makes several interactions with 16S rRNA. Residue G122 is highly conserved, and its mutation to alanine reduces tmRNA-tagging in vitro (14), which suggests that conformational flexibility in this position is essential. Several residues further, a well-defined series of interactions starts with Y126 stacking with 16S G530, which is flipped out into the *anti* conformation. G530 is part of the ribosomal decoding center, and a similar conformation is also observed during recognition of cognate codon-anticodon interaction (20). Y126 is a moderately conserved residue (Fig. 3C), and its mutation alone does not inhibit *trans*-translation (25). However, this position of SmpB is highly restricted to aromatic residues, which implies that the stacking onto G530 is important for tmRNA function. Adjacent to Y126, two highly conserved basic residues are forming ionic bonds with the negatively charged phosphate backbone. K128 interacts with the phosphate group of G530 and R129 with C532. Together, these residues were previously shown to be required for tagging in vivo (14, 19). Starting from residue D132, the sequence then forms an α helix, which extends toward the C terminus and shows weak interactions with 16S rRNA, e.g., with residues K134 and R138. In *E. coli*, the mutation of W147 had a strong effect on tagging efficiency (25), and the corresponding V137 in *T. thermophilus* SmpB, together with L141, may be involved in hydrophobic interactions with F28 and V51 on the surface of ribosomal protein S5. Overall the helical structure of this region is highly conserved and essential for tmRNA-tagging activity

(14, 26). This suggests that these interactions of the carboxy-terminal helix with the ribosome might further stabilize the binding of SmpB to stalled ribosomes. Overall, SmpB appears to recognize stalled ribosomes by making specific interactions with regions of the ribosome that would normally be occupied by mRNA if it were not truncated. After the initial step of *trans*-translation, however, these contacts would need to be disrupted because the ORF of tmRNA would have to replace the C terminus of SmpB and position itself along the mRNA path before translation can resume.

The structure explains why the tmRNA system does not interfere with active protein synthesis. For crystallization, we used an mRNA sequence with two nucleotides in the A site. The second nucleotide is disordered and does

Fig. 3. Interactions of SmpB with the ribosome. (A) The C-terminal tail of SmpB would clash with mRNA downstream of the A-site codon. The mRNA used in this work is colored in magenta, and an extension based on the superposition of a longer mRNA (PDB code 2HGR) (31) is shown in gray. The mRNA nucleotides are numbered starting with the first nucleotide of the A-site codon. (B) SmpB interacts with both the shoulder domain and the 3' major domain of 16S rRNA near the decoding center. (Insets) Close-up views of the interactions near A1492/A1493 and G530 at the decoding center. (C) Sequence alignment for the regions of SmpB interacting with the decoding center with degree of conservation indicated by size. Positively charged residues are highlighted in blue, negative in red, and aromatic in yellow. For details, see SOM and fig. S4.



not interact with SmpB. This ribosome complex mimics the state of a stalled ribosome after cleavage of mRNA by ribosome-dependent nucleases like RelE, which cleaves mRNA in the ribosome after the second nucleotide of the A-site codon (27). Indeed, translating ribosomes treated with RelE are efficient targets of tmRNA during adaptation to starvation conditions (28). Recruitment of tmRNA to ribosomes requires truncated mRNAs, and it has been shown that complexes with more than six nucleotides after the P-site codon are poor substrates for ribosome rescue by tmRNA and SmpB (29). This suggests that tmRNA acts only on ribosomes that have reached the 3' end of a nonstop message or are stalled during translation with subsequent mRNA degradation (30). A superposition of SmpB with a ribosome structure containing a long mRNA (31) shows that the C-terminal tail of SmpB extends into the space normally occupied by mRNA, which leaves space for about four or five nucleotides after the P-site codon (Fig. 3, A and B). The free 3' end of truncated mRNAs probably has a certain degree of structural flexibility, but significantly longer transcripts would ultimately clash with SmpB, which provides a structural rationale for the observation that the tmRNA-SmpB complex does not compete with translation factors for A-site binding during canonical translation (32).

As in normal decoding with tRNA, the tmRNA-SmpB complex facilitates GTP hydrolysis by EF-Tu by inducing a closed conformation of the 30S subunit. During translation, correct codon-anticodon pairing induces conformational changes in the conserved 16S rRNA nucleotides A1492/A1493 and G530 in the decoding center of the 30S subunit, which lead to a closure of the 30S subunit (15, 20, 21). Together with distortions of A-site tRNA, these changes allow the sarcin-ricin loop of the 50S subunit to stabilize an activated catalytic conformation of EF-Tu (33). In the case of tmRNA, there are no codon-anticodon interactions. The tmRNA-SmpB complex shows no major distortions after binding of the ribosome (fig. S2), and SmpB does not interact with ribosomal protein S12, which has a stabilizing role during canonical decoding. However, two key regions of SmpB are close to the critical 16S rRNA bases involved in decoding (Fig. 3B). As discussed earlier, Y126 near the beginning of the carboxy-terminal tail of SmpB stacks with G530. In addition, the decoding bases A1492/A1493 are displaced out of the internal loop in helix 44 but in a conformation that is somewhat different from that observed during canonical decoding (fig. S3). They are close to helix α 1 near the N terminus of SmpB. This region of SmpB is highly conserved (Fig. 3C). N7 and Y14 appear to be important to stabilize the positioning of the α 1 fold between β 1 and β 2, respectively. The charged residues R8, R9, H12, and D13, whose side chains are only weakly ordered, are within bonding distance of A1492 and A1493. These findings

agree with chemical footprinting and hydroxyl radical probing studies, which found a protection of the decoding bases on binding of tmRNA-SmpB to the ribosome (12, 13). However, mutation of the decoding bases did not affect peptidyl transfer to tmRNA in vitro (14). This suggests that regardless of specific interactions with the bases A1492 and A1493, SmpB interacts with the 30S subunit in such a way as to bring the shoulder domain of the 30S subunit (with G530) closer to its 3' major domain (containing A1492 and A1493), which results in a closed conformation that is similar to that induced by cognate tRNA binding (see movie S1). This closed conformation is responsible for an increased rate of GTP hydrolysis in EF-Tu and subsequent accommodation of the 3' end of tmRNA into the peptidyl transferase center (34, 35).

The structure raises some interesting questions about what happens during translocation after accommodation of tmRNA and peptidyl transfer. Translocation must bring the ORF of tmRNA into the path normally occupied by mRNA, so that translation can resume on the ORF. However, in the crystal structure, this mRNA path is occupied by the C terminus of SmpB. Therefore, the C terminus of SmpB has to undergo a major conformational change during translocation. Indeed, in cryo-EM studies of the post-translocated complex of tmRNA, the C terminus of SmpB has been modeled in a different conformation from that seen here, which allows new interactions to take place between SmpB and the sequence upstream of the resume codon (23, 24).

This structure of Ala-tmRNA^{Δm}-SmpB-EF-Tu-GDP bound to a stalled ribosome provides insights into the initial steps of *trans*-translation at near-atomic resolution. SmpB, tmRNA, and EF-Tu together associate in a 1:1:1 stoichiometry only with ribosomes stalled at or near the 3' end of their transcripts. Interactions of SmpB with the decoding center induce a domain closure of the 30S subunit similar to that seen with cognate tRNA, which leads to GTP hydrolysis by EF-Tu. Because the C terminus of SmpB would overlap with the path of normal mRNA, the structure also shows why the tmRNA-SmpB complex can only act on ribosomes stalled on truncated mRNA that extends only a few nucleotides beyond the P-site codon and, thus, would not interfere with normal translation.

References and Notes

1. K. C. Keiler, P. R. Waller, R. T. Sauer, *Science* **271**, 990 (1996).
2. S. D. Moore, R. T. Sauer, *Annu. Rev. Biochem.* **76**, 101 (2007).
3. Y. Komine, M. Kitabatake, T. Yokogawa, K. Nishikawa, H. Inokuchi, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9223 (1994).
4. A. Martin, T. A. Baker, R. T. Sauer, *Mol. Cell* **29**, 441 (2008).
5. A. W. Karzai, M. M. Susskind, R. T. Sauer, *EMBO J.* **18**, 3793 (1999).

6. S. Barends, A. W. Karzai, R. T. Sauer, J. Wower, B. Kraal, *J. Mol. Biol.* **314**, 9 (2001).
7. S. Gutmann *et al.*, *Nature* **424**, 699 (2003).
8. Y. Bessho *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 8293 (2007).
9. K. Cheng *et al.*, *J. Struct. Biol.* **169**, 342 (2010).
10. F. Weiss *et al.*, *RNA* **16**, 299 (2010).
11. S. Kaur, R. Gillet, W. Li, R. Gursky, J. Frank, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 16484 (2006).
12. D. Kurita, R. Sasaki, A. Muto, H. Himeno, *Nucleic Acids Res.* **35**, 7248 (2007).
13. S. Nonin-Lecomte *et al.*, *EMBO Rep.* **10**, 160 (2009).
14. M. R. Miller *et al.*, *RNA* **17**, 1727 (2011).
15. T. M. Schmeing *et al.*, *Science* **326**, 688 (2009).
16. Y. Shimizu, T. Ueda, *J. Biol. Chem.* **281**, 15987 (2006).
17. G. Dong, J. Nowakowski, D. W. Hoffman, *EMBO J.* **21**, 1845 (2002).
18. T. Someya *et al.*, *FEBS Lett.* **535**, 94 (2003).
19. T. R. Sundermeier, D. P. Dulebohn, H. J. Cho, A. W. Karzai, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 2316 (2005).
20. J. M. Ogle *et al.*, *Science* **292**, 897 (2001).
21. J. M. Ogle, F. V. Murphy IV, M. J. Tarry, V. Ramakrishnan, *Cell* **111**, 721 (2002).
22. M. Valle *et al.*, *Science* **300**, 127 (2003).
23. F. Weiss *et al.*, *EMBO J.* **29**, 3810 (2010).
24. J. Fu *et al.*, *EMBO J.* **29**, 3819 (2010).
25. D. Kurita, A. Muto, H. Himeno, *RNA* **16**, 980 (2010).
26. Y. Jacob, S. M. Sharkady, K. Bhardwaj, A. Sanda, K. P. Williams, *J. Biol. Chem.* **280**, 5503 (2005).
27. C. Neubauer *et al.*, *Cell* **139**, 1084 (2009).
28. S. K. Christensen, K. Gerdes, *Mol. Microbiol.* **48**, 1389 (2003).
29. N. Ivanova, M. Y. Pavlov, B. Felden, M. Ehrenberg, *J. Mol. Biol.* **338**, 33 (2004).
30. C. S. Hayes, K. C. Keiler, *FEBS Lett.* **584**, 413 (2010).
31. G. Yusupova, L. Jenner, B. Rees, D. Moras, M. Yusupov, *Nature* **444**, 391 (2006).
32. S. D. Moore, R. T. Sauer, *Mol. Microbiol.* **58**, 456 (2005).
33. R. M. Voorhees, T. M. Schmeing, A. C. Kelley, V. Ramakrishnan, *Science* **330**, 835 (2010).
34. T. Pape, W. Wintermeyer, M. Rodnina, *EMBO J.* **18**, 3800 (1999).
35. M. V. Rodnina, W. Wintermeyer, *Annu. Rev. Biochem.* **70**, 415 (2001).
36. T. M. Schmeing, R. M. Voorhees, A. C. Kelley, V. Ramakrishnan, *Nat. Struct. Mol. Biol.* **18**, 432 (2011).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/335/6074/1366/DC1
Materials and Methods

Figs. S1 to S4

Table S1

References (37–52)

Movie S1

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of the peptidyl-tRNA, consistent with the role of the GGQ motif in peptide release (16, 17).

The C-terminal tail, which is disordered in the nuclear magnetic resonance structure of YaeJ alone (pdb 2JY9), extends from the globular domain and reaches the 30S subunit A site more than 40 Å away (Fig. 1B), which allows it to bind both ribosomal subunits in a manner reminiscent of many ribosome-binding factors. It forms an α helix with the positively charged surface of the tail accommodated inside the mRNA entry channel downstream of the A site. In this position, the basic side chains of the YaeJ tail are free to interact with the phosphate backbone of the ribosomal RNA (rRNA) that forms the walls of the channel. The tail of YaeJ spans the region between the decoding center and the central pseudoknot in the 30S subunit. In this position, it mimics the mRNA in the mRNA entry channel downstream of the A site (Fig. 1B), which is not compatible with the presence of an A-site codon primed for codon-anticodon interaction (Fig. 1C), and this explains why YaeJ does not act on ribosomes during normal translation (4). Because recent biochemical data suggest that YaeJ can catalyze peptidyl-tRNA hydrolysis on ribosomes stalled by a rare codon cluster in vitro (4), the possibility that a codon can occupy the A site cannot be excluded. However, we speculate that the flexibility of the mRNA allows it to avoid a steric clash with YaeJ and still accommodate a few codons downstream from the P site of our model.

Because YaeJ does not have domains that are involved in the recognition of stop codons as in the class I release factors, it uses a different mechanism to bind the ribosome. From previous biochemical data (1, 12), it had been proposed that some rescue factors recognize the empty mRNA entry channel in order to detect stalled ribosomes. Furthermore, nonstop mRNAs and mRNAs with rare codon clusters are cleaved by endonucleases (1, 18), which results in an empty mRNA entry channel, and consequently, ribo-

some rescue is almost always preceded by mRNA cleavage (1, 19). Consistent with these observations, the structure presented here suggests that the YaeJ tail functions as a sensor that probes the occupancy of the mRNA entry channel in order to distinguish between stalled and translating ribosomes. The positioning of the YaeJ tail is also consistent with the proposed position of the similar SmpB tail based on recent biochemical, cryo-electron microscopy (cryo-EM), and homology modeling data on the tmRNA-SmpB rescue system (1, 12, 20, 21). Thus, the structure of YaeJ bound to the ribosome provides direct structural evidence for the use of its tail as a sensor to recognize a stalled ribosome in addition to providing a rationale for the lack of domains required for stop-codon recognition in YaeJ.

The binding of YaeJ to the ribosome induces perturbations in the decoding center and helix 69 (H69) (Fig. 2A). As is seen in standard decoding and in termination that is mediated by stop codons, the binding of the YaeJ tail also induces G530 to switch from *syn* to *anti* conformation by the stacking of Arg¹¹⁸ (R118) on G530 (Fig. 2B), mimicking the stacking normally made by the third nucleotide of the stop codon (22, 23). The positions of A1492 and A1493 in the present complex appear different from those induced by tRNA binding, presumably to avoid a steric clash with the YaeJ tail (Fig. 2C). The side chain of R117 interacts with C518 and Ser⁵⁰ (S50) of ribosomal protein S12 (Fig. 2B) and replaces the interaction that the latter normally makes with position N6 of A1492 during regular decoding (Fig. 2C). In their new orientation, A1492 is stacked within h44 and A1493 stacks with A1913 from H69 (Fig. 2A) to avoid a steric clash with the YaeJ tail (Fig. 2C). The observed stacking between A1493 (h44) and A1913 (H69) was previously reported in the structures of RF1 and RF2 bound to the ribosome (22, 23).

We propose a model based on this structure for how the binding of the YaeJ tail in the mRNA

entry channel influences the accommodation of the GGQ loop in the 50S subunit A site. The binding recognition element (C-terminal tail) and the catalytic GGQ loop of YaeJ are spatially separated by at least 95 Å and span both ribosomal subunits (Fig. 1, A and B). However, biochemical data suggest that binding of the YaeJ tail to the ribosome is required for peptide release (4, 6). Hence, the binding of YaeJ to a stalled ribosome is likely coupled to the rearrangements of its catalytic GGQ loop to ensure efficient rescue. The globular domain and the C-terminal tail of YaeJ are physically connected by a short linker that spans residues 101 to 108. Even though the tail is relatively ordered, some of the residues in the linker region are poorly ordered, as indicated by increased temperature factors and their discontinuous electron density in the $2F_{\text{obs}} - F_{\text{calc}}$ map. On the basis of these observations, it is tempting to suggest that the binding of the YaeJ tail in the mRNA entry channel allows the movement of its globular domain to position the GGQ loop adjacent to the CCA end of the peptidyl-tRNA. Pro¹¹⁰ (P110), which is anchored by its stacking with the conserved A1493 (Fig. 3A), can act as a hinge to fix the conformation of the tail on one side while allowing free movement of the globular domain through the flexible linker. Notably, the presence of a hook like feature at the C terminus of YaeJ (residues 129 to 133), where the tail turns toward the 16S rRNA central pseudoknot (Fig. 3B), appears to stabilize the position of the tail on the other end. This proposed stabilization is consistent with biochemical data showing that deletion of the last 10 residues in YaeJ abolishes its binding to the ribosome (4). Taken together, the structure suggests communication between two distinct regions of the protein where the rigidity conferred by the anchored tail and the flexibility of the linker allow the N-terminal domain to interact with the 50S subunit A site, which results in the proper positioning of the GGQ loop in the PTC primed for catalysis.

The essential GGQ motif containing residues 26 to 28 is positioned in the PTC of the present complex, which is consistent with its involvement in the peptidyl-tRNA hydrolase activity of YaeJ (4, 6). As observed in previous structures of release factors, the unstructured GGQ loop in YaeJ becomes ordered when it binds the ribosome and adopts a short α -helical structure (Fig. 3C) (22–24). The positions of all the residues that are involved in catalysis are identical to their counterparts in the structure of the ribosome in complex with RF2, which suggests a similar mechanism of peptide release (22). The side chain of the catalytic Gln²⁸ was modeled with its carbonyl oxygen within hydrogen-bonding

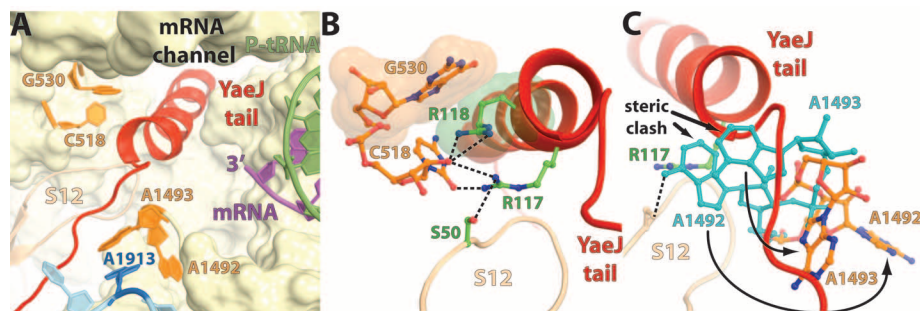
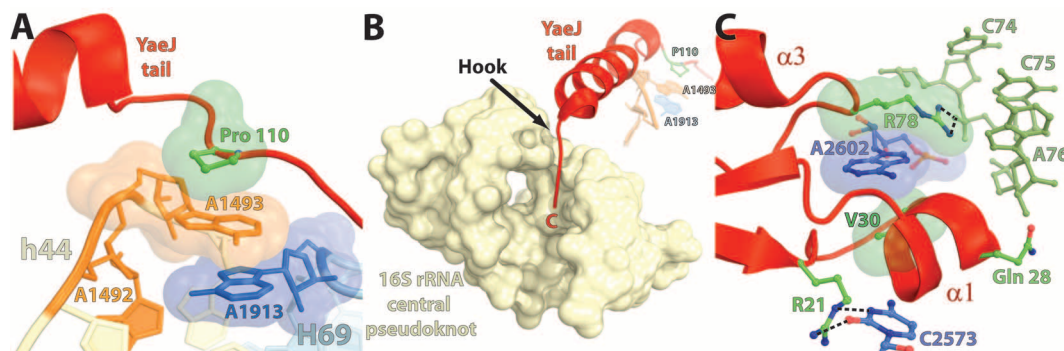


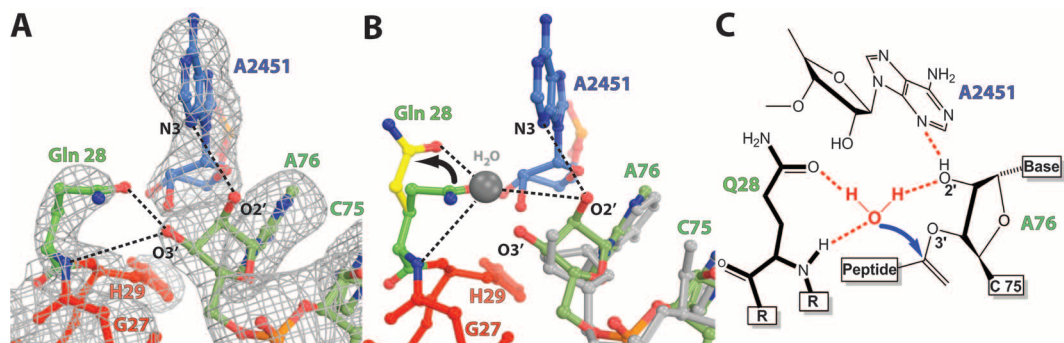
Fig. 2. Interactions of the YaeJ tail with the universally conserved nucleotides in the decoding center. (A) Overview of the YaeJ tail in the decoding center and its interaction with the key bases that are labeled. (B) Close-up view of the stacking between G530 (16S rRNA) and R118 of YaeJ shown as space-filling representation in orange and green, respectively. Putative hydrogen bonds of R117 with C518 (16S rRNA) and S50 from ribosomal protein S12 are shown as black dashes. (C) Conformational differences between A1492 and A1493 from our model (shown in orange) relative to their counterparts from a structure with an A-site-bound tRNA [2J00 (15)] (shown in light blue) reveal that the latter sterically clash with the YaeJ tail. The positioning of these key bases in the present complex is indicated by the curved arrows.

Fig. 3. Stabilization of the YaeJ tail in the mRNA entry channel (A and B) and the GGQ loop in the PTC (C). (A) Stacking between Pro¹¹⁰ (P110) from YaeJ (green), A1493 from h44 (orange), and A1913 from H69 (blue). (B) Interaction of the C-terminal end of YaeJ (residues 129 to 133) with the 16S rRNA central pseudoknot consisting of helix 1 (nucleotides 9 to 13 and 21 to 25) and helix 2 (nucleotides 17 to 19 and 916 to 918). A1493 from h44 in the decoding center (orange), A1913 from H69 (blue), and P110 from YaeJ (green) are shown in the background. The interactions shown in (A) and (B) together anchor the YaeJ tail in the mRNA entry channel. (C) The potential hydrogen bonds are shown as black



dashes. The H-bonds between C2573 and R21, R78, and C75 of the P-tRNA and the stacking between A2602, R78, and V30 (shown as space-filling representation) help to position the GGQ loop next to the CCA end of the P-tRNA.

Fig. 4. Positioning of the GGQ loop and catalysis. (A) σ_A -weighted $2F_{\text{obs}} - F_{\text{calc}}$ electron density map contoured at 1.2σ showing the interactions of the GGQ loop at the site of catalysis. Putative hydrogen bonds between Gln²⁸, A2451, and the ribose of A76 are shown as black dashes. (B) Proposed catalytic mechanism reveals that a small change in dihedral angles of Gln²⁸ as indicated by the arrow (green versus yellow) allows it to coordinate the nucleophilic water [shown as a gray ball modeled based on 1VQ7 (25)] that attacks the aminoacyl ester bond. (C) Schematic representation of the proposed catalysis reaction shown in (B).



distance of the 3'-OH of A76 of the P-site tRNA (Fig. 4A). As proposed earlier (22), a small change in the dihedral angles of the glutamine side chain can accommodate the water molecule that acts as a nucleophile poised for an inline attack on the peptidyl-tRNA ester bond (Fig. 4B). The position of the nucleophilic water can be inferred by a superposition on the structure of a peptidyl-tRNA analog bound to the 50S subunit (Fig. 4B) (25).

The structure presented here supports the following model for YaeJ-dependent rescue of stalled ribosomes. The C-terminal tail of YaeJ functions as a sensor to detect a stalled ribosome based on the occupancy of the mRNA channel. Subsequently, the binding of the YaeJ tail in the mRNA entry channel allows its N-terminal globular domain to sample different orientations through its flexible linker region. A network of interactions between the globular domain of YaeJ and the 50S A site can then stabilize the GGQ loop in the PTC in a conformation that promotes catalysis. After peptide release, the ribosomes may become a substrate for the ribosome recycling factor (RRF), which, together with the elongation factor G (EF-G), dissociates ribosomes into subunits (26). Beyond addressing the critical role of the protein tail in the recognition of a stalled ribosome, this study raises questions about the unassigned roles of extended tails present in many ribosome-binding factors. Together with the recently de-

termined structures of the eukaryotic ribosome (27, 28), our structure reveals the details of how protein segments can mimic RNA to perform important functions. Additional structural studies of other rescue factors are required to establish common architectural features governing the rescue of stalled ribosomes.

References and Notes

1. B. Felden, R. Gillet, *RNA Biol.* **8**, 440 (2011).
2. K. C. Keiler, P. R. Waller, R. T. Sauer, *Science* **271**, 990 (1996).
3. J. Withey, D. Friedman, *J. Bacteriol.* **181**, 2148 (1999).
4. Y. Handa, N. Inaho, N. Nameki, *Nucleic Acids Res.* **39**, 1739 (2011).
5. F. Garza-Sánchez, R. E. Schaub, B. D. Janssen, C. S. Hayes, *Mol. Microbiol.* **80**, 1204 (2011).
6. Y. Chadani, K. Ono, K. Kutsukake, T. Abo, *Mol. Microbiol.* **80**, 772 (2011).
7. Y. Chadani *et al.*, *Mol. Microbiol.* **78**, 796 (2010).
8. E. M. Youngman, M. E. McDonald, R. Green, *Annu. Rev. Microbiol.* **62**, 353 (2008).
9. K. Ito, M. Uno, Y. Nakamura, *Nature* **403**, 680 (2000).
10. R. Richter *et al.*, *EMBO J.* **29**, 1116 (2010).
11. Y. Handa *et al.*, *J. Mol. Biol.* **404**, 260 (2010).
12. D. Kurita, A. Muto, H. Himeno, *RNA* **16**, 980 (2010).
13. D. Kurita, R. Sasaki, A. Muto, H. Himeno, *Nucleic Acids Res.* **35**, 7248 (2007).
14. Materials and methods are available as supporting material on Science Online.
15. M. Selmer *et al.*, *Science* **313**, 1935 (2006).
16. L. Mora *et al.*, *Mol. Microbiol.* **47**, 267 (2003).
17. L. Y. Frolova *et al.*, *RNA* **5**, 1014 (1999).
18. Y. Yamamoto, T. Sunohara, K. Jojima, T. Inada, H. Aiba, *RNA* **9**, 408 (2003).

19. N. Ivanova, M. Y. Pavlov, B. Felden, M. Ehrenberg, *J. Mol. Biol.* **338**, 33 (2004).
20. F. Weis *et al.*, *RNA* **16**, 299 (2010).
21. S. Gutmann *et al.*, *Nature* **424**, 699 (2003).
22. A. Weixlbaumer *et al.*, *Science* **322**, 953 (2008).
23. M. Laurberg *et al.*, *Nature* **454**, 852 (2008).
24. D. E. Burakovsky *et al.*, *RNA* **16**, 1848 (2010).
25. T. M. Schmeing, K. S. Huang, S. A. Strobel, T. A. Steitz, *Nature* **438**, 520 (2005).
26. A. Savelsbergh, M. V. Rodnina, W. Wintermeyer, *RNA* **15**, 772 (2009).
27. J. Rabl, M. Leibundgut, S. F. Ataide, A. Haag, N. Ban, *Science* **331**, 730 (2011).
28. A. Ben-Shem *et al.*, *Science* **334**, 1524 (2011).

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The Transcription Factor c-Maf Controls Touch Receptor Development and Function

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The sense of touch relies on detection of mechanical stimuli by specialized mechanosensory neurons. The scarcity of molecular data has made it difficult to analyze development of mechanoreceptors and to define the basis of their diversity and function. We show that the transcription factor c-Maf/c-MAF is crucial for mechanosensory function in mice and humans. The development and function of several rapidly adapting mechanoreceptor types are disrupted in c-Maf mutant mice. In particular, Pacinian corpuscles, a type of mechanoreceptor specialized to detect high-frequency vibrations, are severely atrophied. In line with this, sensitivity to high-frequency vibration is reduced in humans carrying a dominant mutation in the c-MAF gene. Thus, our work identifies a key transcription factor specifying development and function of mechanoreceptors and their end organs.

Low-threshold mechanoreceptors respond to innocuous mechanical stimulation and are crucial for touch sensation (1, 2). Mechanoreceptors are physiologically and morphologi-

cally diverse and are classified as rapidly adapting, slowly adapting, and D-hair mechanoreceptors (RAMs, SAMs, and D-hairs) on the basis of responses to sustained stimuli and conduction velocities. RAMs/SAMs and D-hairs have large- and medium-diameter myelinated axons, respectively, and are further distinguished by their end-organ morphology. Pacinian corpuscles, Meissner corpuscles, and lanceolate endings are RAMs, whereas Merkel cell-neurite complexes and Ruffini corpuscles are SAMs (3, 4). Distinct mechanoreceptors detect different stimuli, for example, high- or low-frequency vibration, movement, or static skin indentation. Mechanosensory neurons in dorsal root ganglia (DRGs) derive from neural

crest cells, but little is known about their molecular characteristics and development (5, 6). Low-threshold mechanoreceptors and proprioceptors are born during an early neurogenic wave and depend on the basic helix-loop-helix transcription factor Ngn2 for their generation (7). The tyrosine kinase receptor Ret is expressed in early- and late-born neurons (8–10). Recent work showed that “early Ret” neurons are RAMs and depend on Ret for their development (10–12). RAMs also express the transcription factor MafA; however, mutation of MafA has little impact on these receptors (11, 13). The Maf family encodes basic leucine-zipper factors, whose founding member, v-Maf, was identified as a retroviral oncogene (14). We show that c-Maf/c-MAF, a gene known to control eye and lens development (15–18), is crucial for mechanosensory function in mice and humans.

In a screen for genes expressed in the developing murine nervous system, we found c-Maf and MafA coexpressed in mechanosensory DRG neurons (Fig. 1) and lamina III of the dorsal spinal cord [compare (19) with fig. S1A]. c-Maf expression in lumbar DRGs starts around embryonic day 11 (E11), and these “early” c-Maf⁺ neurons coexpress Ret and MafA (Fig. 1A), identifying them as RAMs (10, 11). c-Maf⁺/Ret⁺/MafA⁺ neurons are maintained during development and postnatal life (Fig. 1B and fig. S1, B to D). Around E13, additional sensory neuronal classes begin to express c-Maf (Fig. 1C). Early and late c-Maf⁺ neurons in postnatal DRGs represent 7 and 15% of all neurons, respectively (fig. S1E). All c-Maf⁺ neurons coexpress NF200, indicating that they have myelinated axons (Fig. 1, D). Furthermore, c-Maf expression in DRG neurons is evolutionarily conserved and detectable in chicks and humans (fig. S1, F and G).

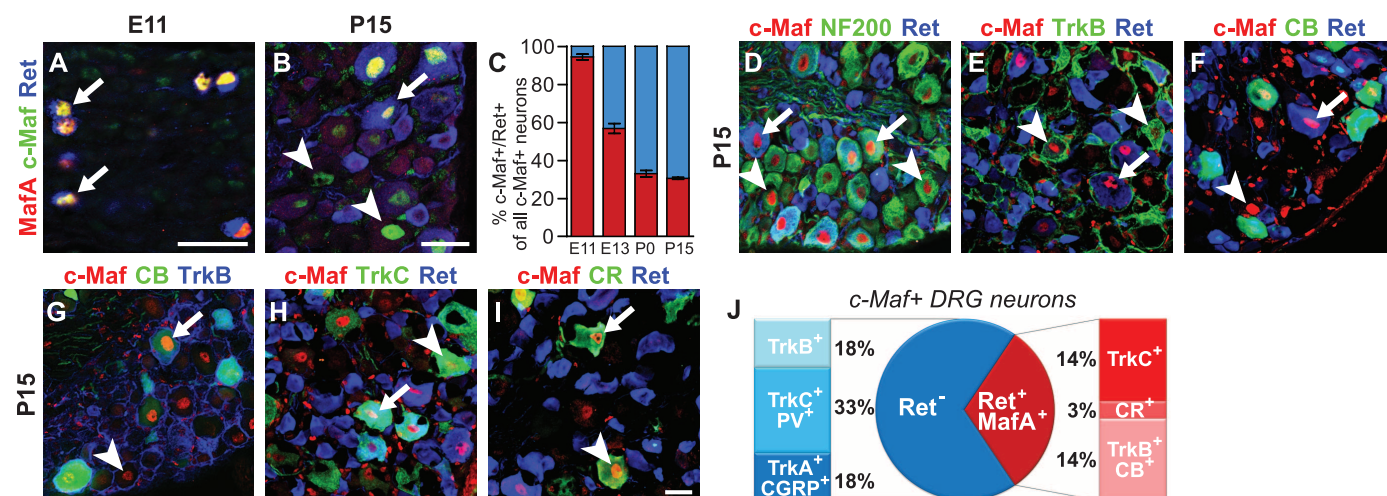


Fig. 1. Sensory neuron classes expressing c-Maf. (A and B) c-Maf, MafA, and Ret expression in lumbar DRG neurons at E11 and postnatal day 15 (P15). (C) Quantification of c-Maf and Ret coexpression at different stages; the vast majority of c-Maf⁺ neurons are Ret⁺ at E11, but subsequently c-Maf⁺/Ret[−] neuronal classes appear. Error bars indicate SEM. (D to I) At P15, all c-Maf⁺/Ret⁺ neurons coexpress

NF200 (D). Further, c-Maf⁺ subpopulations coexpress TrkB and Ret (E), CB and Ret (F), CB and TrkB (G), TrkC and Ret (H), and CR and Ret (I). Arrows and arrowheads point toward c-Maf⁺/CB⁺ and c-Maf⁺/CB[−] neurons, respectively, in (G) and toward c-Maf⁺/Ret⁺ and c-Maf⁺/Ret[−] neurons, respectively, in other panels. (J) Quantification of distinct c-Maf⁺ neuronal populations at P15. Scale bars indicate 30 μ m.

To characterize early c-Maf⁺ neurons, we used a marker panel that defined three c-Maf⁺/Ret⁺/NF200⁺ subtypes on a molecular basis: TrkB⁺/calbindin⁺ (CB), TrkC⁺, and calretinin⁺ (CR), which represent 3, 3, and 1% of all DRG neurons, respectively (arrows in Fig. 1, D to I, and fig. S2). Late c-Maf⁺/NF200⁺ neurons are also heterogeneous, comprising Ret⁺/TrkB⁺ mechanoreceptors, Ret⁺/TrkC⁺/parvalbumin⁺ mechanosensory muscle afferents, and a few Ret⁺/TrkA⁺/CGRP⁺/IB4⁺ nociceptors (arrowheads in Fig. 1, D to I; figs. S2 and S3; summary in Fig. 1J). Furthermore, FABP7⁺ glia also express c-Maf (fig. S3F).

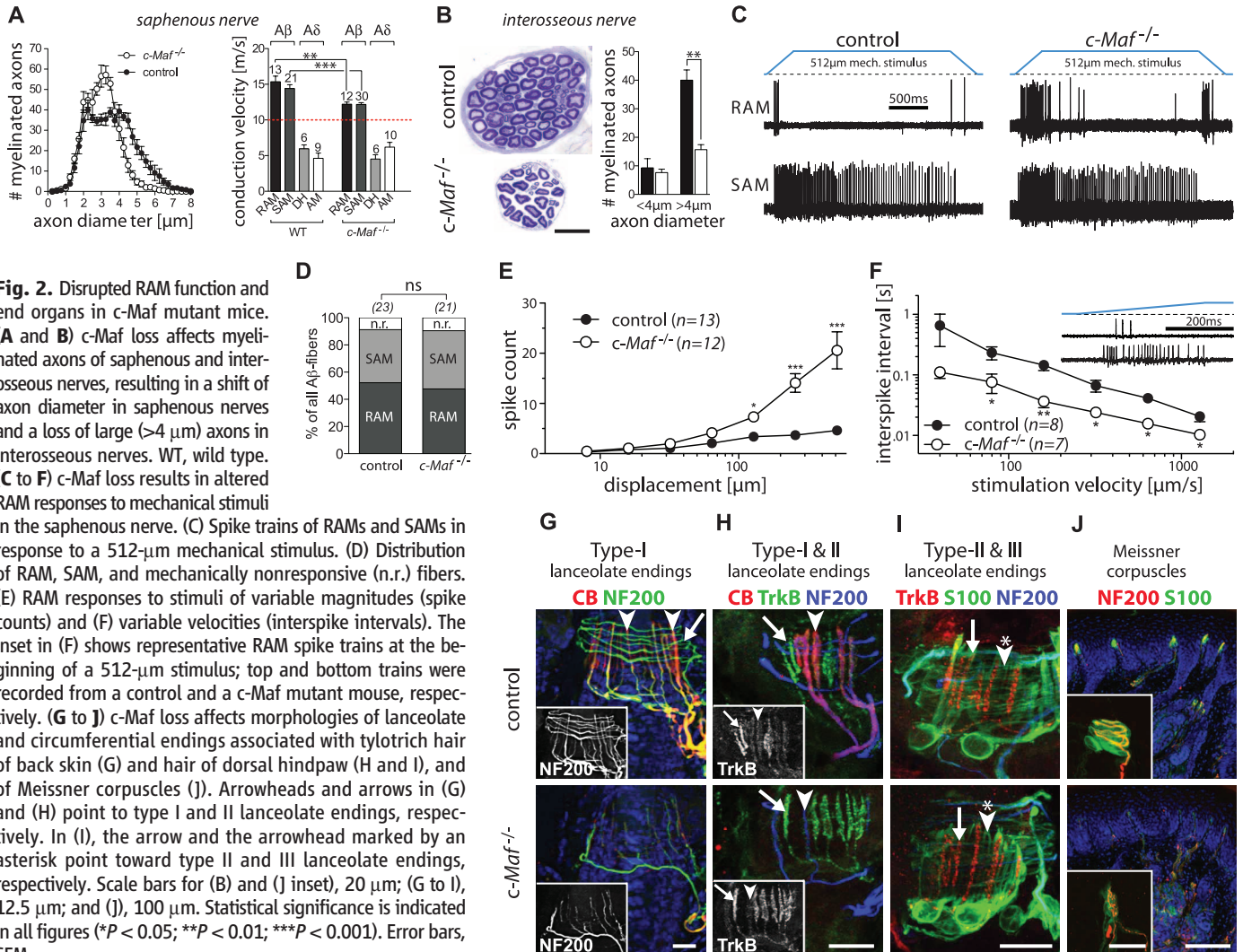
We used mouse genetics to define the role of c-Maf in sensory neurons. Because the null mutation is lethal (15), we generated conditional mutant mice (Isl1^{cre} c-Maf^{flx/flx} mice, hereafter called c-Maf mutants). With use of this strategy, recombination in DRG neurons occurred before c-Maf expression; hence, conditional mutant DRG neurons never expressed c-Maf. However, c-Maf was present in the spinal cord and glia (see fig. S4

for additional information). In c-Maf mutant DRGs, there was no significant change in NF200⁺ neuron quantity. We also assessed the integrity of peripheral nerves. No loss of myelinated axons from the saphenous nerve, a cutaneous nerve rich in mechanoreceptors, was observed (fig. S5, A and B). In c-Maf mutants, axonal size distribution in the saphenous nerve was altered such that large diameter axons became thinner. Myelinated fibers are classified as Aβ (large diameter, fast conducting, >10 m/s) and Aδ (medium diameter, medium conducting, <10 m/s). The shift of axon diameter in the saphenous nerve of c-Maf mutants correlated with a reduced conduction velocity of Aβ but not Aδ fibers (Fig. 2A). Most myelinated axons in the interosseous nerve terminate in Pacinian corpuscles, a scarce mechanoreceptive subpopulation. In contrast to the saphenous nerve, many myelinated axons >4 μm were lost in the interosseous nerve of c-Maf mutants (Fig. 2B).

We next investigated whether the c-Maf mutation alters mechanoreceptor function and used an in vitro skin-saphenous nerve preparation to

record mechanically evoked responses from single nerve fibers (20). In controls, skin indentation evokes RAM firing solely during the onset of stimulus movement. In contrast, RAM firing in c-Maf mutants was prolonged and even continued into the beginning of the static phase. SAMs fired during the entire static phase in controls and c-Maf mutants, and therefore remained distinguishable from mutant RAMs (Fig. 2C and fig. S5, D and E). RAMs and SAMs were present in normal proportions in c-Maf mutants (Fig. 2D). Increased RAM spiking was observed over a wide range of displacement amplitudes (Fig. 2E), and spike intervals were shorter (Fig. 2F). In contrast, firing properties of SAMs were not significantly altered, but their von Frey thresholds were reduced (fig. S5, C to F). D-hair receptors and Aδ nociceptors were unaffected (Fig. 2A and fig. S5, G and H). Thus, the c-Maf mutation profoundly disrupts cutaneous RAM function.

Next, we studied mechanoreceptor end organs in c-Maf mutant skin. We observed that lanceolate endings were heterogeneous with respect



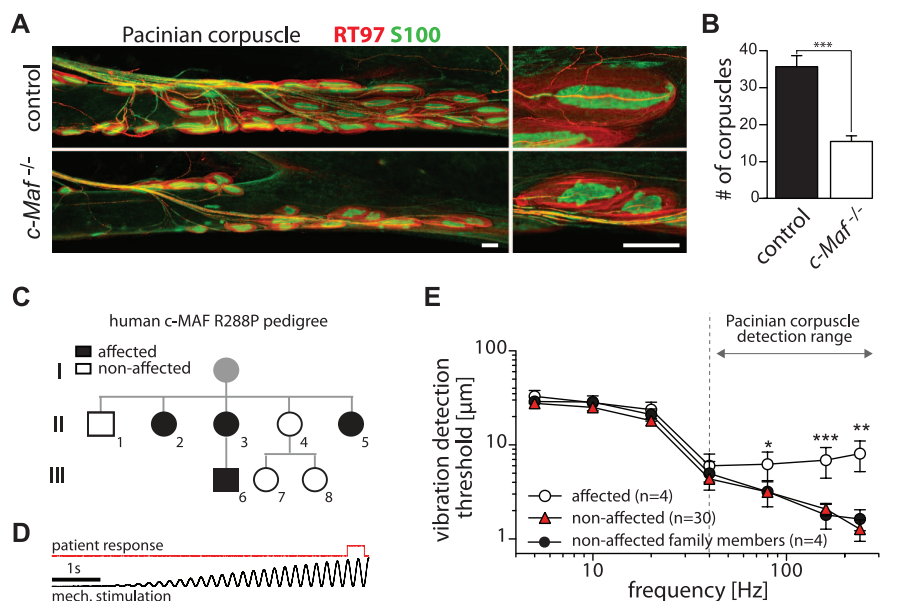


Fig. 3. Pacinian corpuscles in *c-Maf* mutant mice and sensitivity to vibration in patients with dominant *c-MAF* mutation. (A and B) *c-Maf* mutation in mice causes a loss of Pacinian corpuscles and disrupts the morphologies of remaining corpuscles. (C) Pedigree of a family with the R288P *c-MAF* mutation; members carrying the mutant allele (black symbols) suffer from early-onset cataracts. (D and E) Detection threshold of vibrotactile stimuli. (D) Example of a vibration stimulus and response and (E) vibration detection threshold in control and four carriers of the R288P *c-MAF* mutation. In healthy participants and unaffected family members, the detection threshold decreases with increasing frequencies. The detection threshold of affected family members is aberrant at high but not low frequencies. Scale bars, 100 μm. Error bars, SEM.

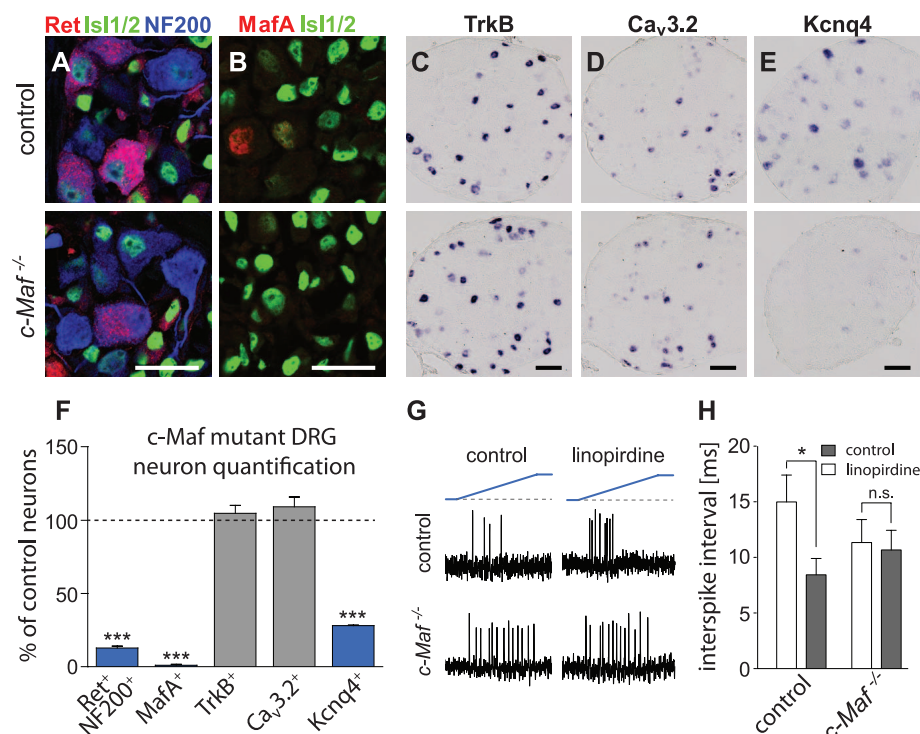


Fig. 4. *c-Maf* and the molecular mechanisms of mechanosensory dysfunction. (A to F) The numbers of RAM neurons that express Ret and MafA and the numbers of neurons that express TrkB, Ca_v3.2, and Kcnq4 were quantified in control and *c-Maf* mutant DRGs. (G and H) Interspike interval of RAMs measured in the skin-nerve preparation of the saphenous nerve in control and *c-Maf* mutant mice in the presence and absence of linopirdine. Representative traces (G) and quantification of interspike intervals (H) are shown. Scale bars for (A) and (B), 30 μm; (C) to (E), 100 μm. Error bars, SEM; n.s., not significant.

to marker expression and *c-Maf*-dependence, indicating functional heterogeneity. We visualized lanceolate endings and associated terminal Schwann cells and defined three neurochemical types: type I (S100⁺/NF200⁺/CB⁺/TrkB^{low}) (arrowheads in Fig. 2, G and H), type II (S100⁺/NF200⁺/CB⁻/TrkB^{high}) (arrows in Fig. 2, H and I), and type III (S100⁺/NF200⁻/CB⁻/TrkB⁻) (asterisks in Fig. 2I) (2I). Type I endings are associated with tylotrich and nontylotrich hair in back skin and with the morphologically distinct dorsal hindpaw hair. Type I lanceolate endings displayed lower levels of NF200 and CB, but not TrkB, in *c-Maf* mutants, but type II and III endings were unaffected (22); the morphology of circumferential endings was also disrupted (Fig. 2, G and H, and fig. S6, B to D). Meissner corpuscles represent an additional type of RAM ending in the glabrous skin and are, like type I lanceolate endings, innervated by axons coexpressing NF200/TrkB^{low}/CB (fig. S6E). In *c-Maf* mutants, the quantity of Meissner corpuscles was reduced by 75% ± 9% and remaining corpuscles appeared rudimentary (Fig. 2J), but the NF200⁺ axons innervating dermal papillae were not lost (8.0 ± 0.2 and 7.1 ± 0.5 innervating axons per section in control and *c-Maf* mutants, respectively). No changes were observed in Merkel cell-neurite complexes (SAMs), muscle spindles, and nociceptive skin endings (fig. S6, F to I).

We next analyzed Pacinian corpuscles, the primary detectors of high-frequency vibration. Pacinian corpuscles are abundant in human palms and fingers, but in rodents they cluster in periosteal (23). In *c-Maf* mutants, the quantity of Pacinian corpuscles associated with fibulae was reduced by 58%, correlating with the axonal loss (61%) in interosseous nerves; remaining corpuscles were small and possessed irregularly shaped S100⁺ cores (Fig. 3, A and B). Thus, four mechanoreceptor classes depend on *c-Maf*: Three terminate in the murine skin (type I lanceolate endings, circumferential endings, and Meissner corpuscles), and the fourth, Pacinian corpuscles, is abundant in murine periosteal. Axonal loss is, however, restricted to Pacinian corpuscle innervation.

In humans, dominant *c-MAF* mutations are associated with ocular developmental abnormalities and cataracts (16–18), but their effects on mechanosensation have not been examined. Encouraged by our results in mice, we tested touch sensitivity in a family comprising four carriers of the dominant Arg²⁸⁸→Pro²⁸⁸ (R288P) *c-MAF* mutation (16) (Fig. 3C). This mutation in the auxiliary DNA binding domain interferes with *c-MAF*-dependent transcriptional activation but does not eliminate *c-MAF* function (24) and represents one of three known cataract-causing point mutations in *c-MAF* (16–18). To assess the function of Meissner and Pacinian corpuscles, we tested vibrotactile acuity over a wide range of frequencies (5 to 240 Hz; Fig. 3, D and E). Pacinian corpuscles are essential for the detection of small-amplitude high-frequency vibrations (25–27). We observed a large increase in the vibration amplitude required to

elicit responses in c-Maf mutant carriers at high but not low frequencies (Fig. 3E). Tactile spatial acuity, that is, the ability to distinguish grids of different spacing, is thought to depend on hair follicle afferents/Meissner corpuscles/Merkel cell-neurite complexes (25, 28, 29) and was not significantly changed (fig. S6J). Thus, the R288P c-Maf mutation interferes with normal vibration detection in humans.

To understand the mechanism underlying mechanoreceptive deficits, we identified changes in gene expression in DRG neurons of c-Maf mutants. Ret and MafA expression was strongly reduced at birth as assessed by counting Ret⁺/NF200⁺ and MafA⁺ neurons (Fig. 4, A, B, and F) and modestly or not affected at E13.5 (fig. S7). Thus, c-Maf is required to maintain but not initiate Ret and MafA expression. We also analyzed DRGs of Ret mutant mice and found that c-Maf expression was unchanged (fig. S7). Thus, c-Maf acts upstream to maintain but not initiate Ret expression during RAM development.

We also examined the expression of receptors and ion channels characteristic of mechanoreceptors like TrkB, Ca_v3.2, and Piezo2, which were unchanged in c-Maf mutants (Fig. 4, C, D, and F, and fig. S7M). Further deregulated genes were identified by using microarray analysis and in situ hybridization. Among these were crystallins (Cryba2 and Crygs), known c-Maf targets encoding structural lens proteins (15). Crystallins also act as chaperones and can impinge on neural physiology. Further, genes encoding the potassium channels Kcng4, Kcnq4, Kcna1, and Kcnh5 were down-regulated in c-Maf mutants (Fig. 4, E and F, and table S1). Kcnq4 was recently found to fine-tune mechanoreceptor function (30). The Kcnq channel blocker linopirdine shortens interspike intervals of RAMs in control but not c-Maf mutants (Fig. 4, G and H), directly implicating Kcnq4 in one aspect of the functional changes that are observed in RAMs of c-Maf mutant mice.

Our analyses show that c-Maf directs mechanoreceptor development. c-Maf acts upstream of Ret, a receptor known to control RAM development, providing a mechanistic basis for this phenotype. c-Maf and MafA are similar in structure and might act redundantly. The fact that MafA expression is not maintained in c-Maf mutant mechanoreceptors can explain the salient function of c-Maf. c-Maf controls many parameters of RAM development, morphology, and function and modulates functional aspects of SAMs. Our analysis also reveals that the most strongly affected RAM subtype in the c-Maf mutant mice are Pacinian corpuscles that specialize in the detection of high-frequency vibration. Pacinian corpuscles and associated axons were largely absent, and residual corpuscles exhibited disrupted morphologies. Because the deformation of the lamellar end organ of Pacinian corpuscles initiates axonal firing (31), remaining corpuscles are expected to be functionally impaired. In line with this, humans carrying a dominant cataract-causing

c-Maf mutation displayed reduced acuity to high-frequency vibration. Other mechanisms like aberrant spinal processing of vibrotactile information might also contribute to this deficit in humans. We conclude that the transcription factor c-Maf directs RAM development and formation of RAM mechanoreceptive end organs.

References and Notes

- Q. Ma, *Neuron* **64**, 773 (2009).
- G. R. Lewin, Y. A. Barde, *Annu. Rev. Neurosci.* **19**, 289 (1996).
- P. Delmas, J. Hao, L. Rodat-Despoix, *Nat. Rev. Neurosci.* **12**, 139 (2011).
- M. Tsunozaki, D. M. Bautista, *Curr. Opin. Neurobiol.* **19**, 362 (2009).
- F. Marmigère, P. Ernfors, *Nat. Rev. Neurosci.* **8**, 114 (2007).
- Y. Liu, Q. Ma, *Curr. Opin. Neurobiol.* **21**, 52 (2011).
- Q. Ma, C. Fode, F. Guillemot, D. J. Anderson, *Genes Dev.* **13**, 1717 (1999).
- D. C. Molliver et al., *Neuron* **19**, 849 (1997).
- I. Kramer et al., *Neuron* **49**, 379 (2006).
- W. Luo, H. Enomoto, F. L. Rice, J. Milbrandt, D. D. Ginty, *Neuron* **64**, 841 (2009).
- S. Bourane et al., *Neuron* **64**, 857 (2009).
- Y. Honma, M. Kawano, S. Kohsaka, M. Ogawa, *Development* **137**, 2319 (2010).
- L. Lecoq et al., *Dev. Neurobiol.* **70**, 485 (2010).
- H. Motohashi, J. A. Shavit, K. Igarashi, M. Yamamoto, J. D. Engel, *Nucleic Acids Res.* **25**, 2953 (1997).
- B. Z. Ring, S. P. Cordes, P. A. Overbeek, G. S. Barsh, *Development* **127**, 307 (2000).
- R. V. Jamieson et al., *Hum. Mol. Genet.* **11**, 33 (2002).
- V. Vanita, D. Singh, P. N. Robinson, K. Sperling, J. R. Singh, *Am. J. Med. Genet. A* **140**, 558 (2006).
- L. Hansen et al., *Invest. Ophthalmol. Vis. Sci.* **50**, 3291 (2009).
- M. Z. Li et al., *Dev. Biol.* **292**, 555 (2006).
- M. Koltzenburg, C. L. Stucky, G. R. Lewin, *J. Neurophysiol.* **78**, 1841 (1997).
- Type II lanceolate endings are TrkB^{high}, and TrkB^{high} neurons do not express c-Maf (<5%). TrkB^{high} neurons coexpress Ca_v3.2 and correspond thus to D-hairs (fig. S6A). Type III lanceolate endings are NF200⁺, and NF200⁺ neurons do not express c-Maf.
- Heterogeneity of lanceolate endings was recently also noted by L. Li et al., *Cell* **147**, 1615 (2011). They report that Aβ-, Aδ-, and C-low threshold mechanoreceptors terminate in lanceolate endings, and comparisons of marker expression indicate that these correspond to type I, II, and III endings, respectively.
- J. Zelená, *Prog. Brain Res.* **43**, 59 (1976).
- N. Rajaram, T. K. Kerppola, *Mol. Cell. Biol.* **24**, 5694 (2004).
- W. H. Talbot, I. Darian-Smith, H. H. Kornhuber, V. B. Mountcastle, *J. Neurophysiol.* **31**, 301 (1968).
- R. S. Johansson, A. B. Vallbo, *J. Physiol.* **297**, 405 (1979).
- K. O. Johnson, T. Yoshioka, F. Vega-Bermudez, *J. Clin. Neurophysiol.* **17**, 539 (2000).
- R. W. Van Boven, K. O. Johnson, *Neurology* **44**, 2361 (1994).
- S. J. Bensmaia, P. V. Denchev, J. F. Dammann 3rd, J. C. Craig, S. S. Hsiao, *J. Neurosci.* **28**, 776 (2008).
- M. Heidenreich et al., *Nat. Neurosci.* **15**, 138 (2012).
- W. R. Loewenstein, M. Mendelson, *J. Physiol.* **177**, 377 (1965).

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Niche and Neutral Effects of Acquired Immunity Permit Coexistence of Pneumococcal Serotypes

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Over 90 capsular serotypes of *Streptococcus pneumoniae*, a common nasopharyngeal colonizer and major cause of pneumonia, bacteremia, and meningitis, are known. It is unclear why some serotypes can persist at all: They are more easily cleared from carriage and compete poorly in vivo. Serotype-specific immune responses, which could promote diversity in principle, are weak enough to allow repeated colonizations by the same type. We show that weak serotype-specific immunity and an acquired response not specific to the capsule can together reproduce observed diversity. Serotype-specific immunity stabilizes competition, and acquired immunity to noncapsular antigens reduces fitness differences. Our model can be used to explain the effects of pneumococcal vaccination and indicates general factors that regulate the diversity of pathogens.

The growing use of multivalent vaccines against antigenically variable pathogens creates a need to understand the individual-

and population-level forces that maintain the coexistence of pathogen strains so that the evolutionary effects of such vaccines can be anticipated. One

of the oldest ideas in ecology is that species are able to coexist by occupying unique niches (1). Stable coexistence involving a shared limiting resource can result when individuals compete more strongly with members of their own species than other species, creating a negative feedback loop that limits growth at high abundance (a stabilizing effect) (2). Among closely related pathogen strains, this kind of niche separation often arises through antigenic variation. By differing in their appearance to the immune system, competing strains effectively subdivide the host population, with each host a “habitat” more favorable to strains not yet encountered and for which strain-specific immunity is lacking. In this way, antigenic types to which few hosts are immune maintain a relative advantage. Niche partitioning through antigenic variation can explain the dynamics of protozoal (3), viral (4), and bacterial (5) pathogen populations and is a core assumption of theoretical models of strain competition (6).

Streptococcus pneumoniae, or pneumococcus, is a ubiquitous pathogen and a leading cause of pneumonia, meningitis, and bacteremia in children worldwide (7). The serotypes of pneumococcus are distinguished by their polysaccharide capsules. Licensed pneumococcal vaccines are serotype-specific and, despite overall benefits, have led to an increase in disease from nonvaccine serotypes. Thus, understanding the forces shaping pneumococcal serotype dynamics is important to interpret the effects of vaccine on disease and predict trends. Serotype-specific (henceforth, “anticapsular”) immunity might in principle provide a mechanism to maintain the diversity of pneumococcal serotypes. To permit coexistence, niche differences must be large enough to overcome differences in fitness that would otherwise lead to competitive exclusion (2). Many biological and epidemiological properties of pneumococcal strains, including properties that contribute directly to fitness, strongly correlate with the thickness of the capsule, which depends on the serotype. Serotypes with thicker capsules tend to have longer durations of nasopharyngeal carriage in young children (8), a competitive advantage in the nasopharynx (9), and a greater ability to escape neutrophil-mediated phagocytosis (10) in vitro, for example, compared with serotypes with thinner capsules. These traits also predict the prevalence of serotypes in carriage (9, 10), which is relatively consistent across human populations (text S1): Serotypes with thicker capsules are relatively more common. Studies of anticapsular immunity have shown, however, that the serotype-specific immunity elicited by natural exposure to pneumococci

is imperfect, frequently allowing repeated colonizations with the same serotype. Three separate studies have found evidence of strong immunity for one serotype (individuals who have carried type 14 are ~90% less likely to carry it again); however, they suggest that such immunity for all other serotypes, if it exists, is much less protective (e.g., point estimates of ≤50% protection) (11–13). We sought to understand how such rel-

atively weak type-specific immune responses could support the coexistence of serotypes with substantial fitness differences.

To do so, we developed an individual-based model (text S2 to S5) constructed to avoid an intrinsic bias toward coexistence (14). We simulated the transmission dynamics of 25 serotypes that differed only in their intrinsic durations of carriage and their in vivo competitive abilities (15, 16).

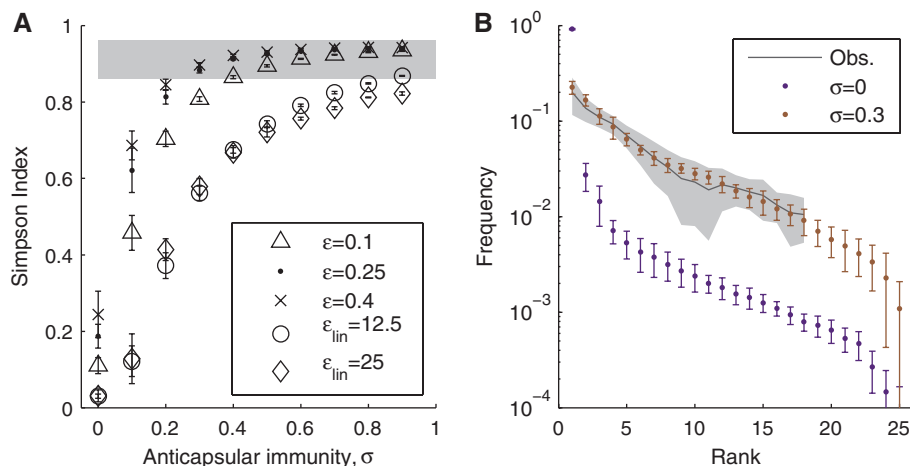
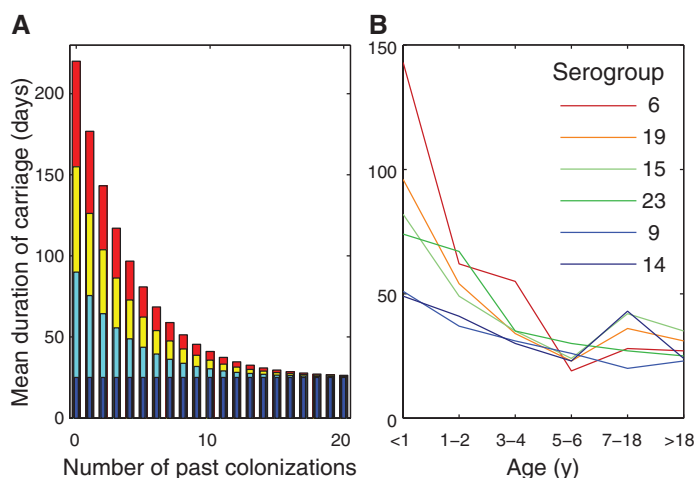


Fig. 1. Serotype diversity is sensitive to the form of acquisition of nonspecific immunity and the strength of anticapsular immunity. **(A)** Diversity, measured by the Simpson index, as a function of the strength of anticapsular immunity for different forms of acquired nonspecific immunity (durations that decay linearly to 0, $\epsilon_{lin} = 12.5$, and $\epsilon_{lin} = 25$; durations that decay exponentially to a nonzero value, $\epsilon = 0.10$, $\epsilon = 0.25$, and $\epsilon = 0.40$; text S4). The Simpson index equals the probability of randomly picking two serotypes from the population with replacement; it was calculated as $1 - D$, where $D = \sum p_i^2$, and p_i denotes the frequency of the i th species. Measures were obtained by averaging annual estimates of the Simpson index for each of the last 20 years of each simulation. The shaded area shows the range of diversity observed in carriage studies. **(B)** Observed (Obs.) rank-frequency distributions from carriage studies and the default model ($\epsilon = 0.25$) without ($\sigma = 0$) and with ($\sigma = 0.3$) anticapsular immunity. The gray line shows the mean distribution from carriage studies, and the shading indicates the SD (text S1). Error bars show SD based on 10 simulations.

Fig. 2. The rate of acquisition of nonspecific immunity was modeled as a linear or exponential function, with the latter supported by observations. **(A)** The duration of carriage of four serotypes modeled as an exponential function of the number of times a host previously cleared any serotype of pneumococcus ($\epsilon = 0.25$). The durations of the different serotypes are shown as superimposed bars, with the color indicating the serotype's fitness (red, most fit; blue, least fit). **(B)** Observed mean durations of carriage of different serogroups (serotypes or groups of antigenically related serotypes) as a function of host age from (8).



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These two components of fitness were positively correlated: Serotypes with longer intrinsic durations of carriage were also better at excluding other serotypes in vivo (9). This competitive ability was modeled as a reduction in a host's susceptibility to a colonizing serotype, with the size of the reduction set by the fitness of any resident serotypes. Anticapsular immunity reduced a host's susceptibility to colonization by a fraction σ if the host had ever previously cleared that serotype (11). This fraction was the same for all serotypes but was varied across simulations. Because anticapsular immunity is thought to result from antibodies, once acquired, it was assumed to persist for the duration of a host's life. To facilitate comparisons of simulations involving different parameter values, we fitted transmission rates so that the total carriage prevalence of pneumococcus in children <5 years old was 40%. Simulations were run for 150 years, and serotype frequencies from the last 20 years were analyzed.

We first considered models with anticapsular immunity and acquired non-serotype-specific immunity (henceforth "nonspecific") that reduced each serotype's duration of carriage (17) by a fixed amount for every past exposure to any serotype (fig. S4). In these cases, the presence of anticapsular immunity, a stabilizing mechanism, promoted serotype diversity (Fig. 1A). However, anticapsular immunity alone was insufficient to overcome differences in serotypes' fitnesses: Even implausibly high levels of immunity (e.g.,

a 90% reduction in susceptibility from prior homologous carriage for each serotype) could not generate the high diversity observed in studies (text S6). Furthermore, high levels of anticapsular immunity in this scenario created an unrealistically low carriage prevalence and diversity in adults (text S6).

Epidemiologic studies of carriage duration (8) suggest that, as individuals acquire nonspecific immunity to pneumococcus, the duration of carriage declines for all serotypes, approaching a fixed value of several weeks in individuals with multiple exposures regardless of serotype (Fig. 2). This mechanism is thought to be largely driven by CD4⁺ T helper-17 cells (18, 19). Modeling this form of nonspecific immunity generated realistic patterns of diversity (Fig. 1 and text S6). A consequence of this nonlinear decline in duration is that, in highly exposed individuals, the fitness advantage of the "best" serotypes is reduced because their durations fall disproportionately (9). Acquired nonspecific immunity thus reduces fitness variation between serotypes, and this reduction is most pronounced in older individuals. This phenomenon might explain the higher diversity of serotypes (in both carriage and invasive disease) in older children and adults (20, 21). Some anticapsular immunity remains necessary in this scenario, underscoring the joint contribution of niche (stabilizing) and neutral (equalizing) mechanisms to coexistence (Fig. 1B) (2). In general, the simulated serotype distributions match observed distributions when

anticapsular immunity confers a 30 to 60% reduction in susceptibility to future colonizations with that type (Fig. 1B and fig. S9). This estimate is consistent with the finding that the confidence intervals of serotype-specific reductions in susceptibility from prior homologous carriage overlap in the range of 34 to 36% (11).

Models with intermediate levels of anticapsular immunity and a nonlinear reduction in the duration of carriage with exposure reproduced other key aspects of pneumococcal dynamics. These models produced relatively consistent rankings of serotype frequencies across populations, a realistic reduction in the duration of carriage with age, higher pneumococcal diversity in adults than in children, and realistic rates of co-colonization (Table 1). Large, multiannual fluctuations in the abundance of rarer serotypes were a common feature in models with anticapsular immunity, and similar fluctuations have been observed in time series of disease isolates (text S6.6) (22).

The major results were robust to the size of the host population, the rate of immigration, and the rate of acquiring nonspecific immunity, and they were modestly affected by host population structure and the total prevalence of pneumococcus (text S6.7 to S6.11). Quantitative validation of our model is limited, however, by the uncertainty in the actual intrinsic durations and transmissibilities of serotypes in nature. Our model assumed that serotypes were

Table 1. Assumptions and constraints of the model and patterns reproduced by the calibrated model. The first section lists the major assumptions of the models and the empirical support for these assumptions. The second section lists the criteria by which the different models of immunity were judged. The

third section lists additional patterns reproduced by the calibrated model ($\sigma = 0.3$, $\epsilon = 0.25$). For each pattern, the supporting figure(s) and the supplementary text sections in which the competing immune models are judged are also listed. Dash entries indicate not applicable.

	Figure	Description/reference
<i>Epidemiologic observations used to define model assumptions</i>		
Weak to nonexistent anticapsular immunity	—	(11–13), text S2
Reduction in the duration of carriage from nonspecific immunity	1	(17–19, 22), text S2 and S4.1
In vivo competition strength correlating with serotype fitness (duration of carriage)	—	(9, 15), text S2
<i>Model outputs used to calibrate parameter values and functional forms</i>		
Total carriage prevalence in children <5 years old	—	Table S1
Diversity (Simpson index)	1A	Main text
Rank-frequency distribution	1B, S9	Text S1 and S6.2
<i>Observed patterns reproduced by the calibrated model</i>		
Stability of rank order	S8, S10	Text S1 and S6.2
Decline in carriage duration with age	S11	text S6.3
Increase in serotype diversity with age	S12	Text S1 and S6.4
Frequency of co-colonizations	S13	Text S6.5
Epidemics of rarer serotypes	S14, S15	Text S6.6
Serotype replacement after vaccination	3, A and B	Main text
Brief increase in diversity after vaccination	3C	Main text

equally infectious per day (in the absence of evidence to the contrary) and had evenly distributed intrinsic durations of carriage. Consistent with theory (2), patterns of diversity were sensitive to the density of serotypes within this range (fig. S16). For example, when we simulated 15 serotypes instead of 25 within the same range, more anticapsular immunity (higher σ) was required to overcome the larger differences in fitness between serotypes. With 35 serotypes, slightly less anticapsular immunity was needed. Our model used a relatively dense packing of fitnesses (text S4.1), implying that the conditions we identify that promote diversity are conservative requirements.

The dynamics of serotypes competing through naturally acquired immunity shed light on serotype replacement after vaccination. Unlike natural colonization, the pneumococcal conjugate vaccine induces anticapsular immunity that is strongly protective against future carriage (23). We expanded our model to include vaccination, assuming that vaccine-induced immunity, like naturally induced anticapsular immunity, permanently reduced susceptibility to future colonizations with specific serotypes. The strengths of natural and vaccine-acquired immunity were varied across simulations. Simulating vaccination with realistic parameters reproduced the observed

rapid and sustained replacement of serotypes targeted by the vaccine (24) when the anticapsular immunity induced by the vaccine was stronger than the naturally occurring form (Fig. 3, A and B). In many scenarios, serotype diversity briefly increased in the few years after vaccination, a trend observed in human populations (Fig. 3C and text S7) (24, 25). In addition, introduction of the simulated vaccine caused the prevalence of total carriage to fluctuate before approaching a new equilibrium (Fig. 3D). In human populations, changes in overall carriage postvaccination have ranged from small increases to large decreases (text S7.2). Whether the simulated serotype diversity or total carriage prevalence ultimately returned to prevaccine levels depended on the ranks of the targeted serotypes (text S7.1). All nonvaccine serotypes increased in frequency after vaccine introduction in approximate proportion to their prevaccine frequencies (Fig. 3E). The precise effects of vaccination on carriage and invasive disease probably depend on the extent of natural versus vaccine-induced cross-immunity between potentially cross-reacting serotypes: Certain serogroups (6, 19, and 23) contain several antigenically similar serotypes, of which only one was in the heptavalent conjugate vaccine (26) (text S7.1). Effects might also depend on the vaccination schedule and rate of introduction and

the efficacy of the vaccine with respect to each serotype.

A theoretically interesting prediction of the model is that sustained reductions in anticapsular and/or nonspecific immunity after vaccination can allow previously excluded serotypes to reinstate successfully if introduced through immigration. If vaccine-induced, serotype-specific immunity to carriage is weaker than that obtained through natural exposure, targeted serotypes can resurge after several years to decades (Fig. 3F). This transient period of low prevalence after vaccination is similar to the “honeymoon period” after a vaccine introduction at a coverage just below what is required to eliminate transmission through herd immunity (27). Instead of arising from unvaccinated individuals, the resurgence here comes from incomplete immunity, particularly a reduction in anticapsular immunity. Even when vaccine-induced immunity is stronger than natural immunity, if total carriage drops precipitously from vaccination, the resulting decline in nonspecific immunity can also permit the return of previously eradicated serotypes (fig. S18).

Previous efforts to model carriage of nasopharyngeal colonizing bacteria have focused on vaccine effects and have assumed, rather than tried to explain, coexistence of serotypes (28–30).

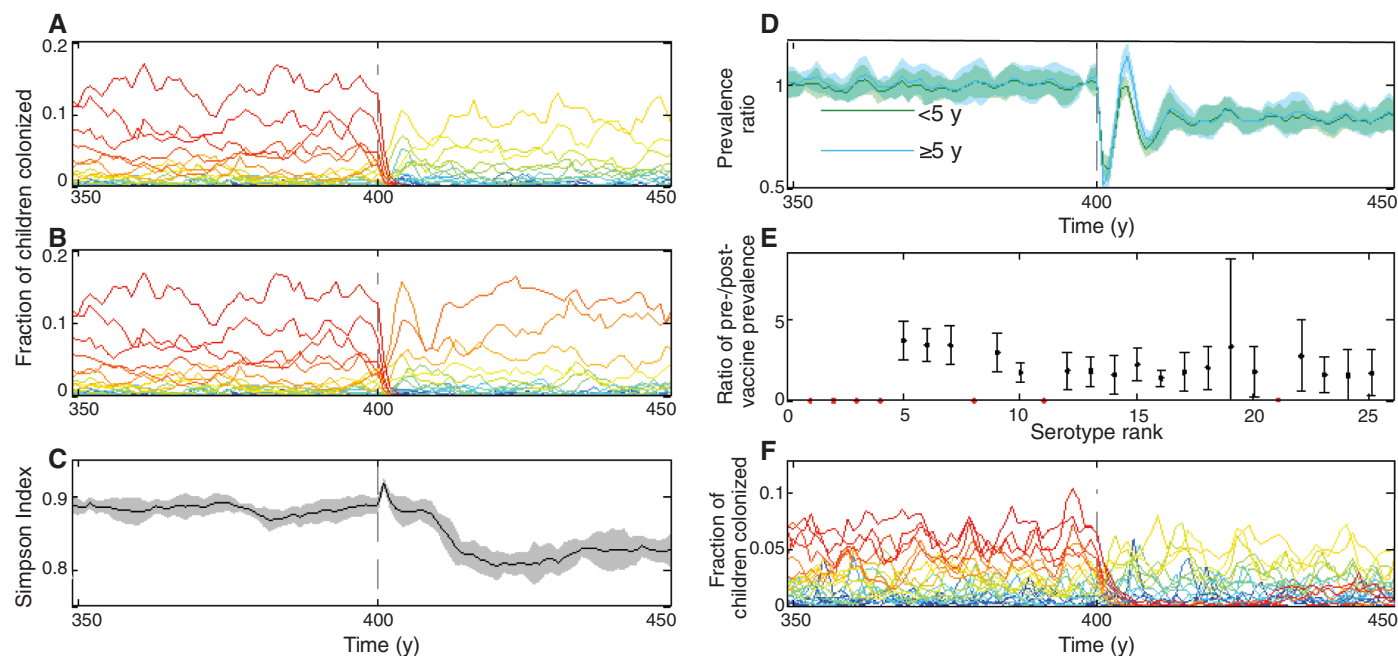


Fig. 3. Vaccination causes at least short-term serotype replacement and can lower total carriage. (A and B) Sample dynamics involving vaccination assuming $\sigma = 0.3$. The prevalences (in children <5 years old) of individual serotypes, colored by their intrinsic fitness as in Fig. 2, are shown. The vaccine was given to all children 6 months old starting in year 400 (dashed line) and had a 60% efficacy against targeted serotypes: (A) Vaccination targeting the seven most intrinsically fit serotypes (ranks 1 to 7). (B) Vaccination targeting serotypes with ranks analogous to those included in the heptavalent pneumococcal conjugate vaccine (ranks 1 to 4, 8, 11, and

21; text S7.1). (C) Simpson index in children <5 years from the scenario in (B). (D) Total carriage prevalence (as a ratio of each year's prevalence to the prevalence in year 399) over time in children <5 years and in individuals ≥5 years from the scenario in (B). (E) The ratio of each serotype's postvaccine prevalence (its mean prevalence in years 410 to 419) to its prevaccine prevalence (years 300 to 399) from the scenario in (B). Targeted serotypes are in red. In (C) to (E), shading or error bars show SD based on 10 simulations. (F) As in (A), vaccination targeting the seven fittest serotypes but with natural immunity ($\sigma = 0.7$) stronger than vaccine-induced immunity.

This study has shown that the diversity of a pathogen can be maintained simply by the interaction of acquired specific and nonspecific immunity, despite fitness differences between serotypes. The sensitivity of the results to the distribution of simulated serotypes' growth rates raises the intriguing question of what upper limits exist for the number of pathogen strains that can coexist stably in a host population and how this depends on the relation between their antigenicity and intrinsic fitness (31). More broadly, this study supports a growing perspective in ecology that recognizes the joint role of niche and neutral dynamics in shaping diversity (2, 32). As antigen-specific vaccines are introduced against pathogens such as human papillomavirus, meningococcus, malaria, and other polymorphic infections, these findings suggest that attention to the details of immune responses may be required to explain prevaccine patterns and make predictions about the effects of vaccines on pathogen populations.

References and Notes

- G. E. Hutchinson, *Quant. Bio.* **22**, 415 (1957).
- P. Chesson, *Annu. Rev. Ecol. Syst.* **31**, 343 (2000).
- C. O. Buckee, P. C. Bull, S. Gupta, *Proc. Biol. Sci.* **276**, 477 (2009).
- K. Koelle, S. Cobey, B. Grenfell, M. Pascual, *Science* **314**, 1898 (2006).
- C. O. Buckee, S. Gupta, P. Kriz, M. C. Maiden, K. A. Jolley, *Proc. Biol. Sci.* **277**, 1635 (2010).
- S. Gupta, N. M. Ferguson, R. M. Anderson, *Science* **280**, 912 (1998).
- K. L. O'Brien *et al.*, *Lancet* **374**, 893 (2009).
- L. Högberg *et al.*, *J. Clin. Microbiol.* **45**, 948 (2007).
- M. Lipsitch *et al.*, *Epidemiology* **10.1097/EDE.0b013e31824f2f32** (2012).
- D. M. Weinberger *et al.*, *PLoS Pathog.* **5**, e1000476 (2009).
- D. M. Weinberger *et al.*, *J. Infect. Dis.* **197**, 1511 (2008).
- P. C. Hill *et al.*, *Clin. Infect. Dis.* **46**, 807 (2008).
- D. Goldblatt *et al.*, *J. Infect. Dis.* **192**, 387 (2005).
- M. Lipsitch, C. Colijn, T. Cohen, W. P. Hanage, C. Fraser, *Epidemics* **1**, 2 (2009).
- M. Lipsitch *et al.*, *Vaccine* **18**, 2895 (2000).
- E. S. Lysenko, R. S. Lijek, S. P. Brown, J. N. Weiser, *Curr. Biol.* **20**, 1222 (2010).
- B. M. Gray, G. M. Converse 3rd, H. C. Dillon Jr., *J. Infect. Dis.* **142**, 923 (1980).
- Y. J. Lu *et al.*, *PLoS Pathog.* **4**, e1000159 (2008).
- R. Malley *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 4848 (2005).
- O. Abdullahi, J. Nyiro, P. Lewa, M. Slack, J. A. Scott, *Pediatr. Infect. Dis. J.* **27**, 59 (2008).
- W. P. Hausdorff, J. Bryant, C. Kloek, P. R. Paradiso, G. R. Siber, *Clin. Infect. Dis.* **30**, 122 (2000).
- Z. B. Harboe *et al.*, *Clin. Infect. Dis.* **50**, 329 (2010).
- H. Rinta-Kokko, R. Dagan, N. Givon-Lavi, K. Auranen, *Vaccine* **27**, 3831 (2009).
- W. P. Hanage *et al.*, *Epidemics* **2**, 80 (2010).
- S. Flasche *et al.*, *PLoS Med.* **8**, e1001017 (2011).
- X. Yu *et al.*, *J. Infect. Dis.* **180**, 1569 (1999).
- A. R. McLean, *Br. Med. Bull.* **54**, 545 (1998).
- M. Lipsitch, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6571 (1997).
- A. Melegaro, Y. Choi, R. Pebody, N. Gay, *Am. J. Epidemiol.* **166**, 228 (2007).
- C. L. Trotter, N. J. Gay, W. J. Edmunds, *Am. J. Epidemiol.* **162**, 89 (2005).
- R. MacArthur, R. Levins, *Am. Nat.* **101**, 377 (1967).
- P. B. Adler, J. Hillerislambers, J. M. Levine, *Ecol. Lett.* **10**, 95 (2007).

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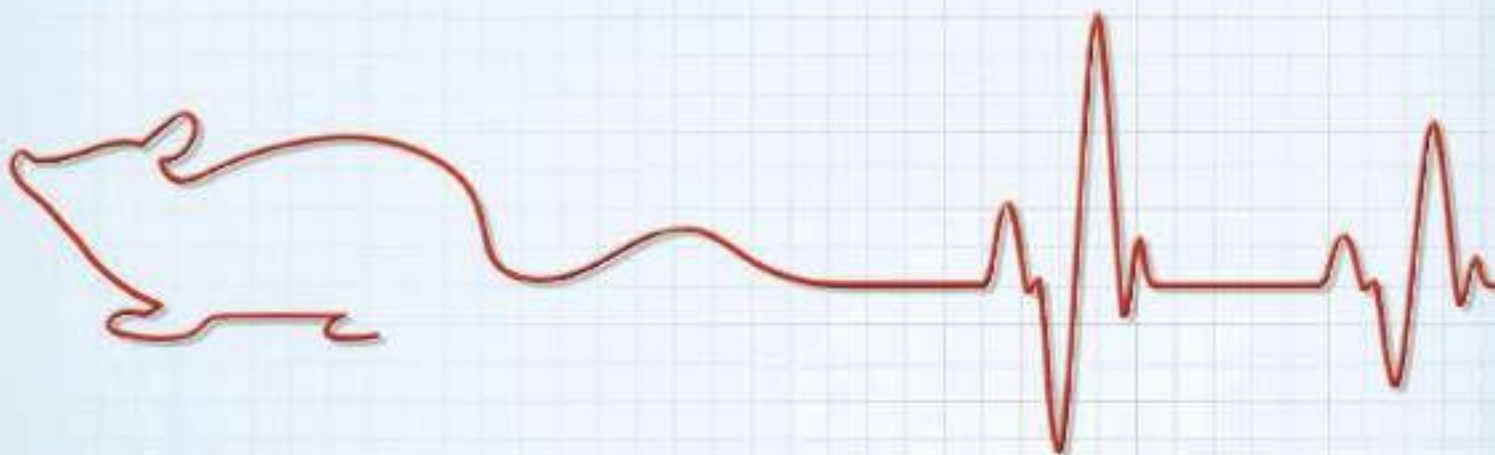
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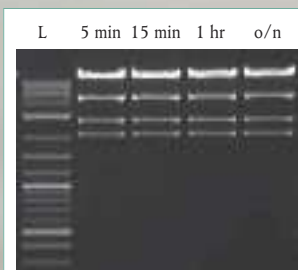
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Polymer Science

Polymer Science Tries to Make It Easy to Be Green

The modern world can't function without plastics, but the planet's environment may not be able to function with them. Can a new generation of polymer chemists rehabilitate these ubiquitous but environmentally troubling materials? **By Alan Dove**



Researchers discovered a simple protocol that grows flexible polymers from soybean oil components, yielding a new plant-based caulk.

Polymer science is a victim of its own success. Its products have become so useful, so successful, and so integral to the modern world that it is difficult to imagine life without them. But while “plastic” has become synonymous with “cheap,” the ubiquity of synthetic polymers comes at a price. Most plastic products are synthesized from nonrenewable petroleum, a few contain compounds that have now become human health concerns, and their environmental longevity means they will spend the overwhelming majority of their life cycles in landfills.

At the same time, polymer science is crucial for solving many of humanity's most pressing problems. New plastic solar cells are already making energy production more sustainable, lifesaving medical devices rely on synthetic polymer coatings, and novel plant-based plastics are pointing the way to a new class of environmentally benign consumer products.

Armed with a plethora of new tools and techniques, polymer scientists are embracing these challenges enthusiastically. A sampling of current work in the field reveals strategies that range from dizzyingly high-tech to surprisingly simple, all with the goal of building better materials with less damage to the environment.

PLASTIC PLANTS

One way to improve polymers' environmental profiles is to make them from renewable feedstocks. While most modern plastics come from petroleum-based starting materials, researchers have known for decades that vegetable oils can also yield high quality polymers. Petroleum, however, is easier to work with; natural feedstocks tend to vary from season to season and place to place, and they don't always have the characteristics chemists like.

“Petroleum feedstocks are very nicely behaved and soluble, but these natural feedstocks, they tend to be somewhat insoluble,” says Atanu Biswas, Ph.D., a research chemist at the **U.S. Department of Agriculture** in Peoria, Illinois. However, Biswas adds that the rising cost of oil and increasing concerns about petroleum mining's environmental impact have prompted researchers to revisit vegetable-based plastics.

Biswas and his colleagues are especially interested in finding polymer starting materials in corn and soybean plants. In one project, for example, the researchers discovered a simple protocol that grows flexible polymers from soybean oil components, yielding a new plant-based caulk. Another effort used vegetable oil to produce cellulose acetate, a common plastic used for everything from eyeglass frames to photographic film.

Regardless of the starting and ending materials, the investigators try to refine their protocols to accomplish the synthesis with as few exotic technologies as possible. “Whatever we have done, mostly we try to do [using] very simple techniques,” says Biswas. Simplicity is not just a philosophical goal, but a crucial factor in making a synthesis scalable; cutting-edge laboratory methods can yield impressive results on the bench, but a straightforward room-temperature reaction is much more likely to work in industry.

That said, Biswas points to two technologies that have had a dramatic impact on vegetable-based plastics: microwaves and ionic solvents. Using microwave reactors, which are essentially very high-tech versions of a home microwave oven, chemists can control the thermal profiles of a reaction very tightly, often drastically boosting the efficiency of a synthesis. “It is intellectually nothing new, but it's really picked up in the last decade,” says Biswas.

Companies such as **Anton Paar** and **Biotage** now offer ready-to-use microwave reactors for both benchtop and industrial synthesis, ensuring that lab-scale protocols can translate easily to a production environment. Meanwhile, **Sigma-Aldrich** sells the Q-Tube pressure reactor, which allows chemists to perform similar types of reactions at research scales without having to invest in a sophisticated microwave system.

Ionic solvents are even simpler, consisting of various molten salts. Though the concept is decades old, chemists have only recently begun to appreciate ionic solvents' ability to dissolve compounds that aren't soluble in water or organic solvents, including many plant materials. Biswas points out that ionic solvents can also be reused after a polymer synthesis, making them “greener” than traditional solvents.

Polymer Science

IN VINO VERITAS

Other polymer chemists are also looking for simple solutions to complicated problems. At the **University of California** in Los Angeles, California, Richard Kaner and his colleagues recently discovered a new way to grow very thin films of nanofiber polymers on a surface. Such thin film coatings often require sophisticated laboratory techniques, but a serendipitous discovery in Kaner's lab revealed a new strategy: shaking up a solution of nanofibers in an organic solvent and letting it sit. Driven by a surface tension phenomenon called the Marangoni effect, the nanofibers climb the inside of the vessel and coat it. First described formally in the 19th century, the same effect generates the "tears" that form inside a glass of wine.

"I prefer processes that are straightforward and scalable, and although they may look simple, there's a lot of chemistry that goes into them," says Kaner. The team has since found that the same surface tension coating technique works well on a variety of surfaces, including flexible plastics. That opens the possibility of making very thin films of conducting polymers into cheap, flexible solar cells, which Kaner and his colleagues are particularly keen to do.

Conducting polymers could also have a wide range of other applications, including forming new catalysts for industrial chemical reactions, high-density computer chips, and implantable medical devices.

Before scientists can create those technologies, though, they need to figure out how to make better conducting polymers. Most modern plastics are highly effective insulators, but research in the 1960s revealed that some polymers, especially polyacetylene, can conduct electricity quite well. Unfortunately, polyacetylene is unstable in air, so chemists have been searching for stable conducting polymers ever since.

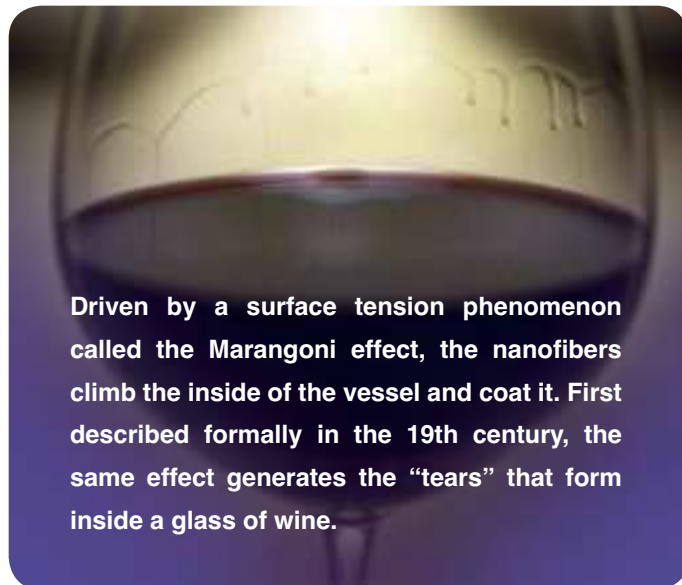
To find those compounds, Kaner's team has been studying materials made from tetramers of aniline, a simple aromatic compound that forms reasonably conductive plastics. "We're trying to understand at a fundamental level what controls conductivity with polymers," says Kaner.

So far, the researchers have found that aligning the chains of the polymer more precisely boosts the material's conductivity. Now they need to find a way to accomplish that reliably. "I would love to see a highly ordered, inexpensive, air stable, water processable conducting polymer that had conductivities that started to approach polyacetylene," Kaner says.

WORKING IN A VACUUM

While the majority of polymer scientists are chemists, the peculiar behavior of some of these compounds has also caught the attention of a few physicists. That's especially true in the area of vapor deposition polymerization, an unusual technique that relies on the unique properties of parylene compounds.

In a typical vapor deposition protocol, researchers vaporize dimers of para-xylylene, then pyrolyze them into monomers before feeding the vapor into a vacuum chamber. The monomers condense onto the surface of an object in the chamber and polymerize into a thin, highly uniform film. "It's really a very unique type of polymer that people use for a number of applications, one is surgical implants, another is electronic packaging," says Toh-Ming Lu, Ph.D., associate director of the **Center for Integrated Electronics at Rensselaer Polytechnic Institute** in Troy, New York.



Driven by a surface tension phenomenon called the Marangoni effect, the nanofibers climb the inside of the vessel and coat it. First described formally in the 19th century, the same effect generates the "tears" that form inside a glass of wine.

Because vapor-deposition polymerization produces such thin polymer layers, it can coat virtually any shape; where other coatings would bridge across small holes or peaks, parylene conforms to them tightly. That's particularly important for surgically implanted devices such as pacemakers and catheters, which must have smooth, non-reactive coverings to prevent corrosion and inflammation. Parylene coatings can also protect electronic circuit boards from dust and moisture, and reduce friction between parts in mechanical systems.

Indeed, vapor deposition polymerization has proven so useful that several companies now specialize in it, providing full-service coating operations for medical researchers and manufacturers. Firms such as **Parylene Engineering** and **SCS Coatings** will take prototypes or whole production runs of a product, coat them and send them back, usually within a week.

While vapor deposition polymerization has become a commodity-priced service for many industries, physicists like Lu are still trying to determine exactly how it works. "Very few people are worrying about this, because people know that you can do this vapor deposition and they just go ahead and apply it," says Lu.

Nonetheless, knowing the mechanism may help researchers push the technology even further. For example, a few years ago scientists discovered that modifying the reaction conditions can cause parylene to grow microscopic peaks and spirals on a surface instead of forming a smooth coating. Theoreticians had thought that such structures were impossible, but Lu and his colleagues have now worked out a plausible mechanism for the reaction. The novel structures may form the basis for a new generation of biosensors that take advantage of the parylene peaks' increased surface area. *continued »*

UPCOMING FEATURES



Innovation in Japan—April 13

Proteomics: Protein Chip Arrays—May 11

Digital Imaging—June 8

Polymer Science

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Specialty Coating Systems

www.scscoatings.com

Asylum Research

www.asylumresearch.com

Sigma-Aldrich

www.sigmaaldrich.com

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U.S. Department of Agriculture

www.usda.gov

Center for Integrated Electronics at Rensselaer Polytechnic Institute

www.rpi.edu/dept/cie

University of California, Los Angeles

www.ucla.edu

Parylene Engineering

www.paryleneengineering.com

University of California, Santa Barbara

www.ucsb.edu

PicoTwist

www.picotwist.com

University of Massachusetts, Amherst

www.mass.edu

BLOCK THAT COPOLYMER

Making polymers form repeating microscopic structures is also a major focus for Thomas Russell, Ph.D., professor of polymer science and engineering at the **University of Massachusetts** in Amherst, Massachusetts. Instead of using parylene, though, Russell and his colleagues prefer a class of structures called block copolymers. "With block copolymers, you take two homopolymers, A and B, that are chemically different, link these together, and ... the system will self-assemble into nanoscopic domains," Russell explains.

The distinct chemical domains are typically a few tens of nanometers across, with structural features that can range from 6 to 100 nm. Because chemists can control the properties of these structures very precisely, they can design materials with a wide range of properties. In one project, for example, Russell's team synthesized a filter that could preferentially concentrate particles of a specific virus for analysis.

Despite the sophistication of the final products, Russell aims to keep their production as simple as possible. "One of the things that I've been trying to do in my career has been to be very lazy in a sense, I want to do as little as possible," says Russell. He points to another project in which his lab etched a block copolymer surface with an electron beam in order to produce nanoscale features suitable for high-density electronic storage devices.

Electron beam lithography is a common tool in the semiconductor industry, but researchers still wanted to make the technique simpler. They discovered that a cut sapphire could serve as a template to generate some of the same features spontaneously, eliminating the need for the electron beam.

Russell and his colleagues are also working on new methods to build polymer-based solar cells. Currently, solar cell development is an embarrassingly empirical process. "Typically what's happened up

to this point is taking component A, mix it with component B, make a device, stick a couple of electrodes on it, measure the efficiency, and there it is," says Russell, adding that "there's a lot of black magic, a lot of art associated with this without a lot of understanding." Using new analytical tools that have become available in the past few years, Russell's team is now trying to understand the principles behind polymer-based solar cells, in order to improve them more deliberately.

A VERY GENTLE TUG

Russell and other polymer scientists cite techniques such as atomic force microscopy (AFM) and new mass spectrometry systems as major drivers of the field. Meanwhile, other scientists are creating the next generation of analytical tools, often borrowing ideas from biologists and physicists in the process.

Microscope designers originally developed AFM in the late 1980s, and the tool became available commercially a few years later from companies such as **Asylum Research**. The instrument uses a minuscule cantilever to scan a microscopic surface physically, like a record needle. Researchers can also use it to manipulate nanometer-scale surface features and pull on individual molecules to measure their strength.

Polymer scientists have been using AFM for several years, but the technique can only measure relatively large forces. To probe the molecular world more sensitively, biophysicists developed a method called magnetic tweezers, which entails attaching magnetic beads to the ends of a polymer and pulling on them with a small electromagnet. But polymer chemists hadn't tried the technique, until a biophysicist stumbled into their field.

"We noticed that single-stranded DNA had a very distinctive elastic response, and that got us started on all this polymer physics," says Omar Saleh, Ph.D., associate professor in the Biomolecular Science and Engineering Program at the **University of California in Santa Barbara**, California. Magnetic tweezers revealed that single-stranded DNA has a nonlinear response to tension, a result that seemed to confirm a decades-old polymer physics theory that no one had been able to test previously.

"No one else can see this scale of polymer response because no one else is measuring with these precise low forces," says Saleh, adding that "this is a good tool to study a lot of different polymers."

As a result of Saleh's data, polymer chemists are now anxious to apply the technique more broadly. Fortunately, they won't have to build their own magnetic tweezer apparatus to do it: **PicoTwist** recently delivered the first off-the-shelf magnetic tweezer systems to researchers in a half-dozen countries.

According to researchers in the field, such new analytical tools are a crucial part of the drive for more environmentally sustainable materials. "If you're going to replace ... the polymers in your plastics with nonpetroleum ones, you have to see what kind of material you're going to be making," says Saleh.

Alan Dove is a science writer and editor based in Massachusetts.

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New Products: Polymer Science

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University of Illinois at Chicago Biochemistry and Molecular Genetics

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DIRECTOR Barshop Institute for Longevity and Aging Studies

The University of Texas Health Science Center at San Antonio (UTHSCSA) seeks a Director of the Barshop Institute, a world-class aging research institute. The Director of the Barshop Institute reports to the Vice President for Research. Candidates in any field related to aging with an outstanding record of scientific achievement, grant support, and mentoring are invited to apply. Dynamic leadership, communication, interpersonal skills, and keen vision are required.

The University of Texas Health Science Center at San Antonio has a long and productive research program in aging. The Barshop Institute's headquarters occupies approximately 40,000 sq. ft. of laboratory and office space in two buildings. The current research strengths of the Barshop Institute include the following areas: the roles of oxidative stress, apoptosis, and mitochondria in aging; cell proliferation and senescence in aging; genomic integrity in aging; insulin/IGF-1 signaling and adiposity in aging; the discovery of interventions for aging-related diseases; the comparative biology of aging; nonhuman primates in aging research; and causes and interventions in neurodegeneration (<http://barshopinstitute.uthscsa.edu/>).

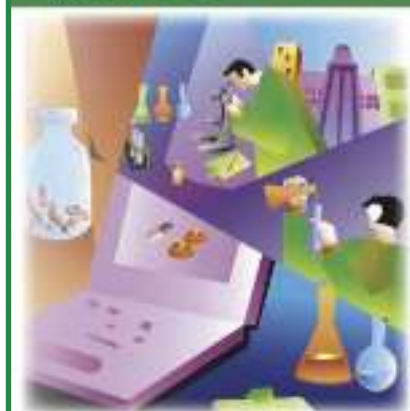
Over 160 faculty members, in all schools and departments of the university, are associated with the Barshop Institute. Barshop Institute-associated faculty have received extramural funding in the range of \$10-14 million per year. Among major grant-related activities of the Barshop Institute are a Nathan Shock Center of Excellence in the Basic Biology of Aging, which has been continuously funded since these centers were initiated in 1995; and a component of the National Institute on Aging's Intervention Testing Centers, which seek to test potential life extension by drug intervention in mice. The Institute is a leader in training; an NIA-supported institutional training grant supports graduate students and postdoctoral fellows in basic aging studies and has a unique program of graduate studies for the Ph.D. degree in the biology of aging. The Director of the Barshop Institute will work closely with the Director of the newly established Center for Healthy Aging to translate the discoveries to our citizens.

Applications should include a Curriculum Vitae, a brief statement of research interests and vision for the institute, and a list of four references. Consideration of applications will begin as they are received and will continue until the position is filled. Send materials electronically to BarshopDirector@uthscsa.edu or by mail to: **Susan L. Naylor, Ph.D., Chair, Barshop Institute Directorship, Office of the Vice President for Research, MC 7763, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900.**

All faculty appointments are designated as security sensitive positions. The University of Texas Health Science Center at San Antonio is an Equal Employment Opportunity/Affirmative Action Employer.

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THE UNIVERSITY OF TENNESSEE HEALTH SCIENCE CENTER

NEUROSCIENCE FACULTY POSITIONS

The Department of Anatomy and Neurobiology (<http://www.uthsc.edu/anatomy-neurobiology/>) at the University of Tennessee Health Science Center seeks outstanding neuroscientists to fill two tenure track positions. Appointments can be at any rank (Assistant, Associate, Full Professor). The department and campus have state-of-the-art laboratories, core facilities, and unique populations of inbred mouse strains for elucidating the neurogenetics of normal and aberrant brain function (<http://cgb.uthsc.edu>). We offer competitive salary and start-up packages, and membership in our vibrant interdepartmental Neuroscience Institute (<http://www.uthsc.edu/neuroscience/>). We seek candidates to complement and extend departmental research on neurogenetics, neuronal excitability and synaptic function, sensory and motor processing, addiction and neurodegenerative diseases (see departmental website). Successful candidates are expected to establish or maintain an independent, extramurally funded research program, and contribute to the department's teaching mission. Candidates must have a Ph.D., M.D., or equivalent. The search will remain open until the position is filled.

For best consideration applications should be submitted by **May 1, 2012**. Submit curriculum vitae, summary of research interests, and contact information for three references, in a single Word or PDF document to bjsmith@uthsc.edu.

The University of Tennessee is an EEO/AA/Title VI/Title IX, Section 504/ADA/ADEA Institution in the provision of its education and employment programs and services.



STANFORD UNIVERSITY SCHOOL OF MEDICINE

The Department of Medicine at Stanford University is recruiting a **Chief for the Division of Immunology and Rheumatology** to lead the research, clinical, and educational activities of the Division. The faculty position is at the Associate Professor or Professor level in the University Tenure Line or Medical Center Line, and the successful candidate should be an accomplished physician investigator with a national/international reputation. Candidates should be Board Certified in Immunology and Rheumatology. Our goal is to develop a premier Division of Immunology and Rheumatology and continue the tradition of outstanding research while building the clinical program. The Chief will be expected to recruit additional faculty to support both laboratory and clinical research and to expand the clinical practice of the faculty. The Chief is expected to strengthen the fellowship program and increase its focus on the research opportunities so prevalent at Stanford. Faculty rank will be determined by the qualifications and experience of the successful candidate.

The Division of Immunology and Rheumatology benefits from an outstanding scientific and clinical environment at Stanford, including active collaborations with the basic science departments and the Stanford Institutes, including a close collaborative relationship with the Institute for Immunity, Transplantation, and Infection.

Candidates should submit a detailed letter of interest, curriculum vitae, and the names of three references to: **Calvin Kuo, MD, PhD, Chair of the Search Committee and Professor of Medicine c/o Gretchen M. Picache, 1070 Arastradero Rd, Rm 255, Palo Alto CA 94304, Mail Code:5850; gpicache@stanford.edu.**

Stanford University is an Equal Opportunity Employer and is committed to increasing the diversity of its faculty. It welcomes nominations of and applications from women and members of minority groups, as well as others who would bring additional dimensions to the university's research, teaching and clinical missions.



EXECUTIVE DIRECTOR

START DATE: JULY 1, 2012

Columbia University's new Mind Brain Behavior Initiative (MBBI) is exploring the mechanisms and logic of brain function, with the aim of linking brain activity to the workings of the mind, and to the nuances of human behavior in both health and disease. The Initiative is active now, and will soon have its intellectual home in the Jerome L. Greene Science Center, a 450,000 sq. ft. interactive space, designed by the Renzo Piano Building Workshop and located on the University's new 16-acre Manhattanville Campus in the heart of New York City. The Jerome L. Greene Science Center will be the research base for 70 independent scientific laboratories, and will house a state-of-the-art brain imaging facility, a mind-brain education center and a small psychiatry/neurology outpatient clinic. Occupancy of the Jerome L. Greene Science Center will begin early in 2016.

MBBI'S EXECUTIVE DIRECTOR will have a critical role in the academic programming of the Jerome L. Greene Center, and in integrating new research themes with existing University programs, schools, centers and departments. Additional responsibilities will include planning and implementing educational outreach programs, and establishing close ties with translational and clinical neuroscience efforts within the University. Working in close collaboration with MBBI senior scientific leadership, the Executive Director will oversee a core management team, will coordinate MBBI's strategic planning process, and will ensure the effective implementation of scientific objectives.

Qualifications: Prior experience in academia, and/or a leadership role in an academic research, governmental, or relevant private sector organization. Prior experience managing complex academic organizations with substantial budgets is essential, as are demonstrated skills in managing a large and closely-knit team. An M.D., and/or PhD in biomedical sciences or a related field is needed. Compensation is competitive and will be commensurate with experience and achievement. The successful applicant should have a clearly defined record of scientific accomplishment, but the primary responsibilities for this position will emphasize organizational leadership, not scientific pursuit.

Columbia University is an Equal Opportunity/Affirmative Action Employer.

Please contact Warren E. Ross, M.D., c/o betsy.messina@kornferry.com



Director of the Melville Laboratory for Polymer Synthesis

Department of Chemistry
£64,379 pa

Applications are invited for the post of Director of the Melville Laboratory for Polymer Synthesis in the Department of Chemistry, to take up appointment on 1 October 2012 or as soon as possible thereafter.

The Melville Laboratory is an interdisciplinary unit, which, though based in the Department of Chemistry, enjoys strong links with many Departments and research groups throughout the University and worldwide. The Director is expected to provide leadership in the Laboratory's research programmes, and to foster links with other research centres. Candidates must have an international reputation in their field of specialisation, which can be in any branch of polymer synthesis. The successful candidate will be expected to contribute to the undergraduate and postgraduate teaching programmes of the Department of Chemistry.

Applications should include a CV, publications list, a statement (up to 8 pages) of research interests and future plans, contact details for three professional referees, and a completed form CHRIS/6 Parts I and III (downloadable from <http://www.admin.cam.ac.uk/offices/hr/forms/chris6/>), and should be sent electronically to Dr Howard Jones, Department of Chemistry, Lensfield Road, Cambridge CB2 1EW (email: hrnj1@cam.ac.uk). Informal enquiries about the post may be addressed to Professor Daan Frenkel (df246@cam.ac.uk).

Information about the Melville Laboratory's activities may be found at <http://www-melville.ch.cam.ac.uk> and further information about the Department of Chemistry may be found at <http://www.ch.cam.ac.uk>

Quote Reference: MA13502. Closing date: 13 April 2012.

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THE UNIVERSITY OF HONG KONG

 香港大學

Founded in 1911, The University of Hong Kong is committed to the highest international standards of excellence in teaching and research, and has been at the international forefront of academic scholarship for many years. The University has a comprehensive range of study programmes and research disciplines spread across 10 faculties and about 100 sub-divisions of studies and learning. There are over 23,400 undergraduate and postgraduate students coming from 50 countries, and more than 1,200 members of academic and academic-related staff, many of whom are internationally renowned.

Post-doctoral Fellowships and Research Assistant Professorships

Applications are invited for a number of positions as Post-doctoral Fellow (PDF) and Research Assistant Professor (RAP), at the University of Hong Kong, on or before February 28, 2013. Appointments will be made for a period of 2 to 3 years.

PDF and RAP posts are created specifically to bring new impetus and vigour to the University's research enterprise. Positions are available from time to time to meet the strategic research needs identified by the University. Positions are available in the following Faculties/Departments/Schools/Centres:

• Urban Planning and Design	• Microbiology
• School of Humanities (Linguistics)	• Orthopaedics and Traumatology
• Faculty of Dentistry	• Pathology
• Faculty of Education	• Psychiatry
• Computer Science	• Centre for Reproduction, Development and Growth
• Electrical and Electronic Engineering	• Stem Cell and Regenerative Medicine Consortium
• Mechanical Engineering	• Surgery
• Law	• Chemistry
• Biochemistry	• Physics
• Centre for Cancer Research	• School of Biological Sciences
• Community Medicine	• HKJC Centre for Suicide Research and Prevention
• Research Centre of Heart, Brain, Hormone and Healthy Aging	• The State Key Laboratory of Brain and Cognitive Sciences
• Research Centre of Infection and Immunology	• The State Key Laboratory of Liver Research
• Centre of Influenza Research	
• Medicine	

Post-doctoral Fellows

PDFs are expected to devote full-time to research. Applicants should be doctoral degree holders having undertaken original research that has contributed to the body of knowledge. A highly competitive salary commensurate with qualifications and experience will be offered. Annual leave and medical benefits will also be available.

Research Assistant Professors

The main focus of an RAP's duty is research. RAPs can however be assigned some teaching duties, up to 50% of the normal teaching load. Applicants should be research active and have a proven publication record. A highly competitive salary commensurate with qualifications and experience will be offered, with a contract-end gratuity and University contribution to a retirement benefits scheme (totalling up to 15% of basic salary). Annual leave, and medical/dental benefits will also be offered.

Procedures

Prospective applicants are invited to visit our webpage at <http://jobs.hku.hk> to view the list of the Faculties/Departments/Schools/Centres and their research areas for which PDF/RAP positions are currently available. Before preparing an application, they should contact the Head of the appropriate academic unit to ascertain that their research expertise matches the research area for which a vacant PDF/RAP post is available.

Applicants must submit a completed University application form, which should clearly state **which position** they are applying for; and in which **academic discipline**. They should also provide further information such as details of their research experience, publications, research proposals, etc.

Application forms (341/1111) can be obtained at <http://www.hku.hk/apptunit/form-ext.doc>. Further particulars can be obtained at <http://jobs.hku.hk/>. **Closes April 21, 2012.** The University thanks applicants for their interest, but advises that only shortlisted applicants will be notified of the application result.

The University is an equal opportunity employer and is committed to a No-Smoking Policy

POSITIONS OPEN



FACULTY POSITION

Biochemistry and Molecular Biology

The Department of Biochemistry and Molecular Biology at Drexel University College of Medicine invites applications for tenure-track faculty positions at the **ASSISTANT, ASSOCIATE, or FULL PROFESSOR** level. Individuals whose research complements existing strengths in the department, including cancer biology, DNA replication and repair, DNA damage signaling, transcription, apoptosis, cell cycle, yeast genetics, cytokines, and membrane proteins are encouraged to apply. Research laboratories are conveniently located in Center City Philadelphia, which offers a collegial and stimulating environment with many opportunities for collaboration. The Department also includes faculty from the Fox Chase Cancer who participate in the graduate programs. The Department occupies recently renovated space, including new state-of-the-art facilities for imaging, protein analysis (mass spec and biosensor) and crystallography; generous startup funds are available. Successful candidates will have a Ph.D. and/or M.D., relevant postdoctoral experience, and a strong record of research accomplishments. Faculty are expected to establish vigorous, independent, and well-funded research programs and to participate in graduate and medical education.

The Drexel University College of Medicine is the nation's largest private medical school. Drexel University has 17,000 students and more than 1,300 faculty members and is ranked among the top 100 universities in the nation, and has recently been named as one of the top "Up-and-Coming" national universities in the 2011 U.S. News College Rankings.

To apply, please submit a single PDF containing curriculum vitae, statement of research interests, and names of three references to **e-mail: lucia.boyer@drexelmed.edu**; please include the words "Biochemistry and Molecular Biology Search" on the subject line.

TWO FACULTY POSITIONS

Columbia University Medical Center

The Columbia University Center for Motor Neuron Biology and Disease (MNC) and the Eleanor and Lou Gehrig MDA/ALS Research Center seek up to two new Faculty with interests in motor neuron biology and disease, especially amyotrophic lateral sclerosis (ALS). The successful candidate(s) will join a translational program that brings together basic, genetic, clinical, and epidemiological research (**websites: <http://www.ColumbiaMNC.org>; <http://www.cumc.columbia.edu/dept/als>; <http://www.cumc.columbia.edu/dept/sergievsky>**).

For the first position, we are particularly interested in hiring a clinician-scientist who can see ALS patients and in parallel carry out his/her own research program in this area. However, nonclinical candidates with a strong translational program are also invited to apply.

For the second position, we seek a candidate with a strong track record in epidemiology and clinical genetics who is motivated to work on ALS and related disorders.

Attractive startup packages will be offered. As members of the MNC, faculty will have access to core facilities, including high throughput screening and internal grant programs.

Columbia University has a world-renowned program in neurobiology and behavior and in medical and surgical neurology. Faculty will be affiliated with the Departments of Neurology and/or Pathology and Cell Biology and will interact with other programs and centers, including the Gertrude H. Sergievsky Center.

To apply online, please go to **website: <https://academicjobs.columbia.edu/applicants/Central?quickFind=54755>**.

Columbia University takes Affirmative Action toward Equal Employment Opportunity.

POSITIONS OPEN

PHYSIOLOGY POSITION in the Colleges of Veterinary Medicine and Medicine

The Colleges of Medicine and Veterinary Medicine at the University of Illinois at Urbana-Champaign invite applications for a tenure-track position at the **ASSISTANT or ASSOCIATE PROFESSOR** level. We seek candidates with an established or strong potential for developing an independent, innovative research program in an area broadly encompassing cardiovascular physiology. Candidates will have the opportunity to form collaborative, interdisciplinary relationships with a research faculty strong in bioengineering, biomedical imaging, stem cell research, aging, neuroscience, nutritional science and translational science (**website: <http://biomedical.illinois.edu/>**) as well as interface with the local medical community including the Carle Heart and Vascular Institute (**website: <http://carle.org/MedicalServices/Heart.aspx>**).

The successful applicant will have demonstrated teaching experience and be expected to provide cardiovascular physiology instruction to professional students at both Colleges. The position will be based within the Department of Comparative Biosciences, College of Veterinary Medicine (**website: <http://vetmed.illinois.edu/cb/>**) with a joint appointment in the College of Medicine (**website: <http://www.med.illinois.edu/>**).

The position is a full-time, nine-month tenure-track appointment, available September 2012. Candidates must possess a Ph.D., DVM, M.D., or equivalent. Candidates with postdoctoral or advanced research training are preferred. Salary and rank will be commensurate with qualifications.

Qualified applicants should electronically submit a cover letter, a statement of research and teaching experience, curriculum vitae, and contact information for three references to Illinois Human Resources (**website: <https://jobs.illinois.edu>**). For inquiries, contact Dr. David Bunick, search chair, at **e-mail: dbunick@illinois.edu** or **telephone: 217-333-2318**.

In order to ensure full consideration, applications must be received by May 15, 2012.

The University of Illinois is an Affirmative Action/Equal Opportunity Employer and welcomes individuals with diverse backgrounds, experiences, and ideas who embrace and value diversity and inclusivity ([website: <http://www.inclusiveillinois.illinois.edu>](http://www.inclusiveillinois.illinois.edu)).

ASSISTANT/ASSOCIATE PROFESSOR Tenure Line Stanford Division of Hematology

The Division of Hematology in the Department of Medicine at Stanford University School of Medicine is recruiting a tenure-line faculty member at the Assistant or Associate Professor level. The successful candidate will have an outstanding record (or the promise of same) of research in the broad area relevant to hematology and will be expected to successfully compete for research funding. The predominant criterion for appointment in the University Tenure Line is a major commitment to research and teaching. Candidates should have an M.D. or M.D.-Ph.D. and be hematologist and/or oncology board certified or eligible.

Applicants should send curriculum vitae, statement of research interests, and the names and addresses of three references to:

Linda M. Boxer, M.D., Ph.D.
Chief, Division of Hematology
Stanford University
269 Campus Drive
CCSR Building, Room 1155
Stanford, CA 94305-5156
E-mail: lboxer@stanford.edu

Stanford University is an Equal Opportunity Employer and is committed to increasing the diversity of its faculty. It welcomes nominations of and applications from women and members of minority groups, as well as others who would bring additional dimensions to the University's research, teaching, and clinical missions.

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